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The natural variation allele *OsGSW3.2* in *Oryza rufipogon* is involved in brassinosteroid signaling and influences grain size and weight

Xiaoyu Liang[1,2,3,†], Yang Liu[1,2,4,†], Min Tian[1,2,3], Weixun Jiang[1,2,3], Yuebin Zheng[1], Zhixiong Chen[1,2,3], Xiangdong Liu[1,2,3,*] and Lan Wang[1,2,3,*]

[1]*State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou 510642, China,*

[2]*Guangdong Provincial Key Laboratory of Plant Molecular Breeding, South China Agricultural University, Guangzhou 510642, China,*

[3]*Guangdong Base Bank for Lingnan Rice Germplasm Resources, College of Agriculture, South China Agricultural University, Guangzhou 510642, China, and*

[4]*Pharmaceutical Engineering Institute, College of Humanities & Information Changchun University of Technology, Changchun 130122, China*

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*For correspondence (e-mail wanglan@scau.edu.cn and xdliu@scau.edu.cn).
†These authors contributed equally to this work.

SUMMARY

Oryza rufipogon **is the ancestor of cultivated rice and harbors many elite genes; thus, this plant is an important germplasm for improving rice varieties. Grain size is an important factor in determining rice yield and quality. In this study, we identified a natural variation allele from the *O. rufipogon* inbred line Huaye3 (HY3), which is located on chromosome 3 and named it *GRAIN SIZE and WEIGHT* 3.2 (*OsGSW3.2*). The *OsGSW3.2* knockout (KO) mutant presented increased grain size and weight, which was associated with decreased chlorophyll content and long awns. The overexpression of $OsGSW3.2^{HY3}$ caused a significant decrease in grain size and weight. *OsGSW3.2* negatively regulates grain size through cell proliferation. Transcriptomic analysis of spikelet hulls from the KO lines and wild-type HY3 revealed that the differentially expressed genes (DEGs) were enriched mainly in plant–pathogen interactions, plant hormone signal transduction, and the plant MAPK signaling pathway, and so on. A laminar inclination experiment verified that *OsGSW3.2* was involved in the BR signaling pathway. Yeast two-hybrid, BiFC, LAC, and pull-down experiments verified that OsGSW3.2 interacted with OsGSK4, which was related to BR signaling, in yeast and plant cells. OsGSW3.2 influenced rice grain size and weight via interaction with OsGSK4. Haplotype analysis of a core collection of cultivated rice revealed that transcriptional accumulation and differential SNPs in the coding region may influence grain size and weight. Our results provide new insight into the role of *OsGSW3.2* in affecting grain size and weight, which will help elucidate the genetic basis of rice domestication.**

Keywords: ***Oryza rufipogon*****, natural variation, *OsGSW3.2*, grain size, domestication.**

INTRODUCTION

Cultivated rice from Asia was domesticated from the wild rice species *Oryza rufipogon* approximately 9000 years ago in China (Fornasiero et al., 2022). Many morphological traits and physiological characteristics, such as longer grains and no or only short awns, changed markedly during domestication. Today, cultivated rice has become one of the major edible crops in the world and feeds half of the human population worldwide. Grain size is an important trait that affects yield and quality in rice. Currently, there are more than 600 genes related to rice grain size on the 12 chromosomes of rice, and at least 22 grain size-related quantitative trait loci (QTLs) related to natural variation have been isolated; however, very few of these cloned grain size genes have been derived from wild rice (Jiang et al., 2022; Kang et al., 2020). Wild rice contains a vast reservoir of traits important for the genetic improvement of modern cultivars, such as seed size, plant architecture, and seed shattering (Fornasiero et al., 2022; Hasan et al., 2023; Izawa, 2022).

Several studies have revealed that many signaling pathways, mainly the G protein signaling pathway, ubiquitination pathway, mitogen-activated protein kinase (MAPK) signaling pathway, phytohormone signaling pathway, transcriptional regulator signaling pathway, and oleoresin lactone signaling pathway, are involved in the regulation of rice grain size (Li et al., 2019). In the plant MAPK signaling pathway, the MAPK cascade generally consists of three or more kinases, namely, MAPKs, MAPK kinases (MAPKKs), MAPKK kinases (MAPKKKs), and MAPKKK kinases (MAPKKKKs), which play important roles in several signal transduction pathways in plants (Chen et al., 2021). *DWARF AND SMALL GRAIN 1* (*DSG1*) encodes the MAPK OsMAPK6, which has phosphorylating activity, and DSG1 influences rice grain size by interacting with OsMAKK4 (Liu, Hua, et al., 2015). *GRAIN SIZE AND NUMBER 1* (*GSN1*) acts as a negative regulator of the MAPK cascade in coordinating the trade-off between grain number and grain size (Guo et al., 2018). OsMKK4 is phosphorylated by OsMKKK10, which phosphorylates OsMAPK6, and OsMKKK10-OsMKK4-OsMAPK6 acts as a cascade to promote grain growth and activates OsMAPK6 to regulate grain size by promoting cell proliferation in spikelet hulls (Xu et al., 2018). In contrast, ERECTA 1 (OsER1) acts upstream of the cascade signal OsMKKK10-OsMKK4-OsMPK6 to negatively regulate the number of grains per spike in rice through the regulation of cell division and participates in rice spike morphogenesis, constituting the OsER1-OsMKKK10-OsMKK4-OsMPK6 signaling pathway (Guo et al., 2020).

Phytohormones play important roles in plant growth and development. Importantly, auxin and brassinosteroids (BRs) affect grain size mainly through the regulation of the cell cycle. *BIG GRAIN 1* (*BG1*) is involved in the auxin signaling pathway that regulates cell division and expansion. Compared with those of the wild type, the plant height, panicle length, grain length, and grain width increased to different degrees after the overexpression of *BG1*, and the degree of phenotypic changes was positively correlated with the expression level of *BG1* (Liu, Tong, et al., 2015). *THOUSAND-GRAIN WEIGHT 6* (*TGW6*) encodes IAA-glucose hydrolase, which has an important effect on the thousand-grain weight trait in rice. Alleles of *tgw6* in Nipponbare control cell proliferation and grain length by regulating the level of IAA. Loss of *tgw6* allele function in Kasalath produces pleiotropic changes in the source organ, resulting in increases in grain weight and yield (Ishimaru et al., 2013). *BRASSINOSTEROID INSENSITIVE 1* (*OsBRI1*, *D61*) encodes a Leu-rich repeat (LRR) receptor-like kinase involved in BR signaling that plays multiple roles in rice growth and development. Compared with those in the wild type, *d61* mutations block the BR signaling pathway and are insensitive to externally applied BR, as evidenced by shorter plant heights, reduced leaf inclination, shorter leaf sheaths, and impediments to dark morphology construction (Guan et al., 2020; Tian et al., 2023).

The molecular mechanism of grain size regulation is very complex, and the regulatory pathways involved are diverse. *GRAIN WIDTH 2* (*GW2*) negatively regulates rice grain width by ubiquitinating WG1 to regulate the expression of *WIDTH GRAIN 1* (*WG1*) and *OsbZIP47* (Hao et al., 2021). The GRAIN WIDTH 5 (GW5) protein is a positive regulator of oleoresin lactone signaling and can inhibit the kinase activity of GLYCOGEN SYNTHASE KINASE 2 (GSK2), affecting the accumulation of BRASSISTEROID RESISTANT 1 (BZR1) and consequently affecting grain width and grain weight (Liu et al., 2017; Zhang et al., 2021). Epigenetic modifications and methylation can also affect grain development. For example, hypomethylation of the promoter region related to the *ABSCISIC ACID INSENSITIVE 3 (ABI3)/VIVIPAPOUS1 (VP1) 6* (*RAV6*) gene leads to a small grain size in *Epi-rav6* mutants (Zhang et al., 2015). F-box E3 ubiquitin ligase 206 (FBX206) acts as a negative factor in BR signaling and regulates grain size and yield in rice via interactions with OVATE family protein 8 (OsOFP8) and OsOFP9 (Sun et al., 2024).

In our previous studies, we colocalized 37 genes related to traits via BSA-seq analysis using an F_2 segregation population derived from a cross of the *O. rufipogon* inbred line Huaye 3 (HY3) and the cultivated variety KJ01 (Wang et al., 2022). Among them, the *LOC_Os03g43030.1* gene, derived from the wild rice inbred line HY3, has two exons and one intron. The cellular component of *LOC_Os03g43030.1* is intracellular membrane-bound organelles (GO:0043231) and cytoplasmic parts (GO:0044444) according to the GO annotation, and it encodes an extensin (precursor) in SwissProt_annotation and a putative uncharacterized protein in TrEMBL_annotation. Here, we describe the biological function of *LOC_Os03g43030.1*, a gene named *OsGSW3.2*, which influences grain size and weight via interaction with *OsGSK4* related to BR signaling. Our studies provide new insight into the role of *OsGSW3.2* in grain size determination and help us understand the genetic basis of rice domestication.

RESULTS

Expression profiling and subcellular localization of OsGSW3.2

The difference in the coding sequence of *OsGSW3.2* between the parents of Huaye 3 (HY3) and KJ01 generated seven SNPs, three of which resulted in amino acid variation (Table S1). The promoter region of *OsGSW3.2* between HY3 and KJ01 also produced eight SNPs in the 1000 bp region upstream of the initiation codon (Table S2). *OsGSW3.2* is a constitutively expressed gene that is expressed in all tissues, including the roots, stems, leaves, and panicles, with relatively greater transcript accumulation in the leaves,

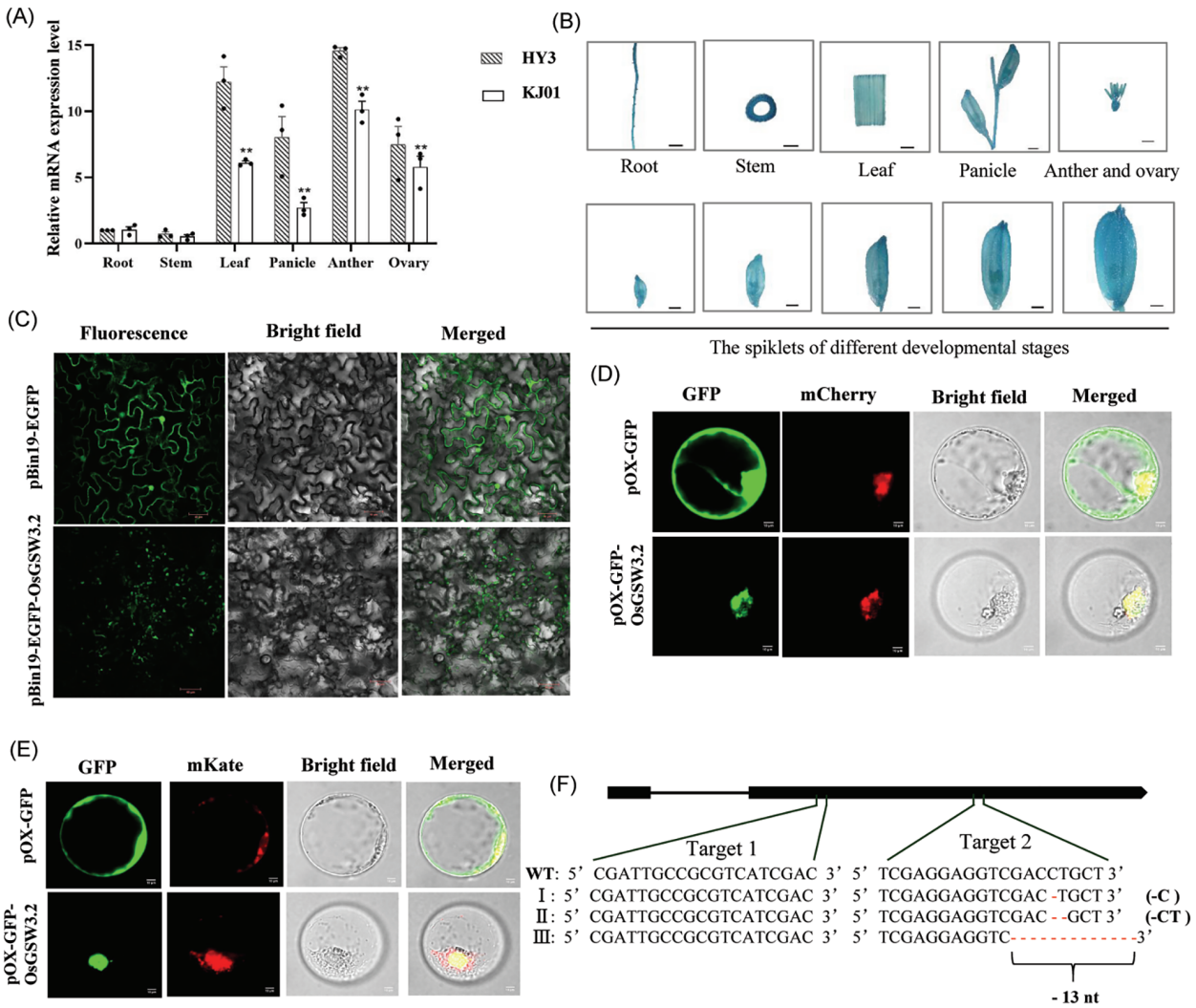


Figure 1. Expression pattern and subcellular localization of OsGSW3.2 in plants.
(A) *OsGSW3.2* was expressed in all the tissues of HY3 and KJ01 according to RT–qPCR ($n = 3$). The data are presented as the means ± SDs, and Student's *t* test was used to determine the *P* values. $*P < 0.05$, $**P < 0.01$.
(B) Histochemical analysis of GUS activity in different tissues and at different developmental stages of grains. The scale bars represent 1 mm.
(C) pBin19-EGFP-OsGSW3.2 and pBin19-EGFP were transformed into *Nicotiana benthamiana* leaves and transiently coexpressed in *N. benthamiana* leaf epidermal cells. The scale bars represent 40 μm.
(D) pOX-GFP-OsGSW3.2 (OsGSW3.2 and green fluorescent protein fusion protein) and mCherry (Golgi localization sequence-fused mCherry protein) were cotransformed into rice protoplasts. Fluorescence in the Golgi was visualized via confocal microscopy, and the scale bars represent 10 μm.
(E) pOX-GFP-OsGSW3.2 (OsGSW3.2 and green fluorescent protein fusion protein) and mKate (ribosome localization sequence-fused mKate protein) were cotransformed into rice protoplasts. Fluorescence was visualized in ribosomes via confocal microscopy, and the scale bars represent 10 μm.
(F) The three editing types of the two target sites of *OsGSW3.2* for the KO lines were shown. I, II, and III indicate type I, type II, and type III, respectively.

panicles, anthers, and ovaries (Figure 1A). In general, the expression level of *OsGSW3.2* was greater in HY3 than in KJ01. To characterize the *OsGSW3.2* expression patterns further, the activity of the $OsGSW3.2^{HY3}$ promoter was studied via β-galactosidase (GUS) in the Nipponbare background. GUS activity was detected in the roots, stems, leaves, and spikelets at different developmental stages, anthers, and ovaries, and the results were consistent with those of the RT–qPCR analysis (Figure 1B).

To explore the localization of the OsGSW3.2 protein in subcellular organelles, OsGSW3.2 was fused with the green fluorescent protein GFP to generate the subcellular localization vector OsGSW3.2-EGFP, after which the fusion protein was used to infect the epidermal cells of *Nicotiana benthamiana* leaves. Confocal laser scanning microscopy was used to observe the expression of the fusion protein in the epidermal cells of *N. benthamiana* leaves. The results revealed that OsGSW3.2 was localized in the

cytoplasm (Figure 1C). To further verify the subcellular fraction, we constructed pOX-GFP-OsGSW3.2 and Golgi-mCherry, as well as pOX-GFP-OsGSW3.2 and RPL27AB-mKATE fusions driven by the 35S promoter. The two groups of fusion proteins were subsequently transferred into rice protoplasts, and the results revealed that the pOX-GFP-OsGSW3.2 fusion protein was localized in the Golgi body (Figure 1D) and ribosomes (Figure 1E) of the cytoplasm.

OsGSW3.2 affects multiple agronomic traits and negatively regulates grain size and weight

To research the biological function of *OsGSW3.2*, by designing two targets in the coding region, *OsGSW3.2* was knocked out in the HY3 background via clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) editing technology. A total of twelve transgenic plants were obtained. Compared with the wild-type HY3 plants, the transgenic plants presented the variant phenotype, with significant increases in grain size, panicle length, plant height, and 1000-grain weight but significant decreases in the number of tillers and chlorophyll content, with very long awns attached at the tips of the seeds. The two target sites of *OsGSW3.2* in these transgenic plants were subsequently sequenced. In general, three editing types (type I, type II, and type III) were generated at the two target sites (Figure 1F). In the first target site, no base was changed among the three editing types. In the second target site, three different editing modes were generated: one base deleted in type I, two bases deleted in type II, and 13 bases deleted in type III. The three editing types all caused a shifted-code mutation in *OsGSW3.2*. These T_0 knockout (KO) plants were subsequently planted into T_1 lines, but only those with type I plants grew, and T_1 seeds were harvested. The variant phenotype of the T_1 plants was the same as that of the T_0 plants, and the variation was stably inherited in the offspring (Table S3; Figure 2A,B). *OsGSW3.2* is an important gene for rice growth that affects multiple agronomic traits, including grain size, chlorophyll content, and awn development. The mRNA expression level of *OsGSW3.2* in the KO lines was slightly lower than that in the wild-type HY3 plants (Figure 2C).

Here, the grain size variety of the KO lines was further analyzed. Compared with that of wild-type HY3 plants, the grain length of the KO lines increased by 16.12%, and the grain width increased by 11.91%, which led to a 1000-grain weight increase of 32.46%. The yield of each KO plant after the awns of the grains were removed was subsequently investigated, and the results revealed an increase of 48.31% relative to that of the wild-type HY3 plants (Figure 2D).

To further study the function of *OsGSW3.2*, $OsGSW3.2^{HY3}$ was overexpressed in the HY3 background. Thirteen transgenic plants were obtained, ten of which presented obvious differences in grain size (Table S4). Among them, two overexpressed mutants (OE-1 and OE-6) were planted into T_1 lines to further investigate the variant phenotype, and the results indicated that these variations maintained stable inheritance in the progeny (Figure 2A,B). The mRNA expression levels of *OsGSW3.2* in the OE-1 and OE-6 lines were much greater than those in the wild-type HY3 (Figure 2C).

These results indicated that *OsGSW3.2* is a domesticated rice gene that suppresses awn development, reduces chlorophyll content, and negatively regulates grain size and weight.

OsGSW3.2 influences grain size via cell proliferation in spikelet hulls

Spikelet hulls have an important effect on rice grain shape. To explore the cytological mechanism through which *OsGSW3.2* influences grain size, the outer epidermal cells of the spikelet glumes of the wild-type HY3, KO, and OE-1 lines were observed by scanning electron microscopy (Figure 3A). Compared with that of the wild-type HY3, the cell length of the outer epidermis of the KO spikelet glumes was significantly shorter, and the cell width was obviously narrower; this increased the number of cells in the longitudinal and transverse directions of the KO spikelet glumes. Compared with that of wild-type HY3, the cell length of the outer epidermis of the OE-1 lines was slightly shorter, but there was no obvious difference in cell width. Therefore, the difference in the cell number of spikelet glumes in the longitudinal and transverse directions was not obvious between the OE-1 lines and wild-type HY3 (Figure 3B).

The transverse sections of the central part of the spikelets in the KO lines were observed via plastic semithin sectioning by microscopic observation (Figure 3C). In the KO lines, the average single-cell area clearly decreased, and the number of cells in the transverse section significantly increased (Figure 3D). These results were consistent with those of the scanning electron microscopy observations. In conclusion, *OsGSW3.2* influences grain size by suppressing cell proliferation in hulls.

Transcriptomic analysis

To further understand the molecular mechanism by which *OsGSW3.2* influences grain size, we performed transcriptome analysis (RNA-seq) of young spikelet hulls derived from the wild-type HY3 and KO lines. Additionally, 25% and 50% full-length hulls of the mature grains from the wild-type HY3 and KO lines were selected to identify the gene regulatory network of *OsGSW3.2*. At the 25% full-length hulls of grains (FLG) stage in the HY3 and KO lines, 2307 differentially expressed genes (DEGs) were detected, 860 of which were upregulated and 1447 of

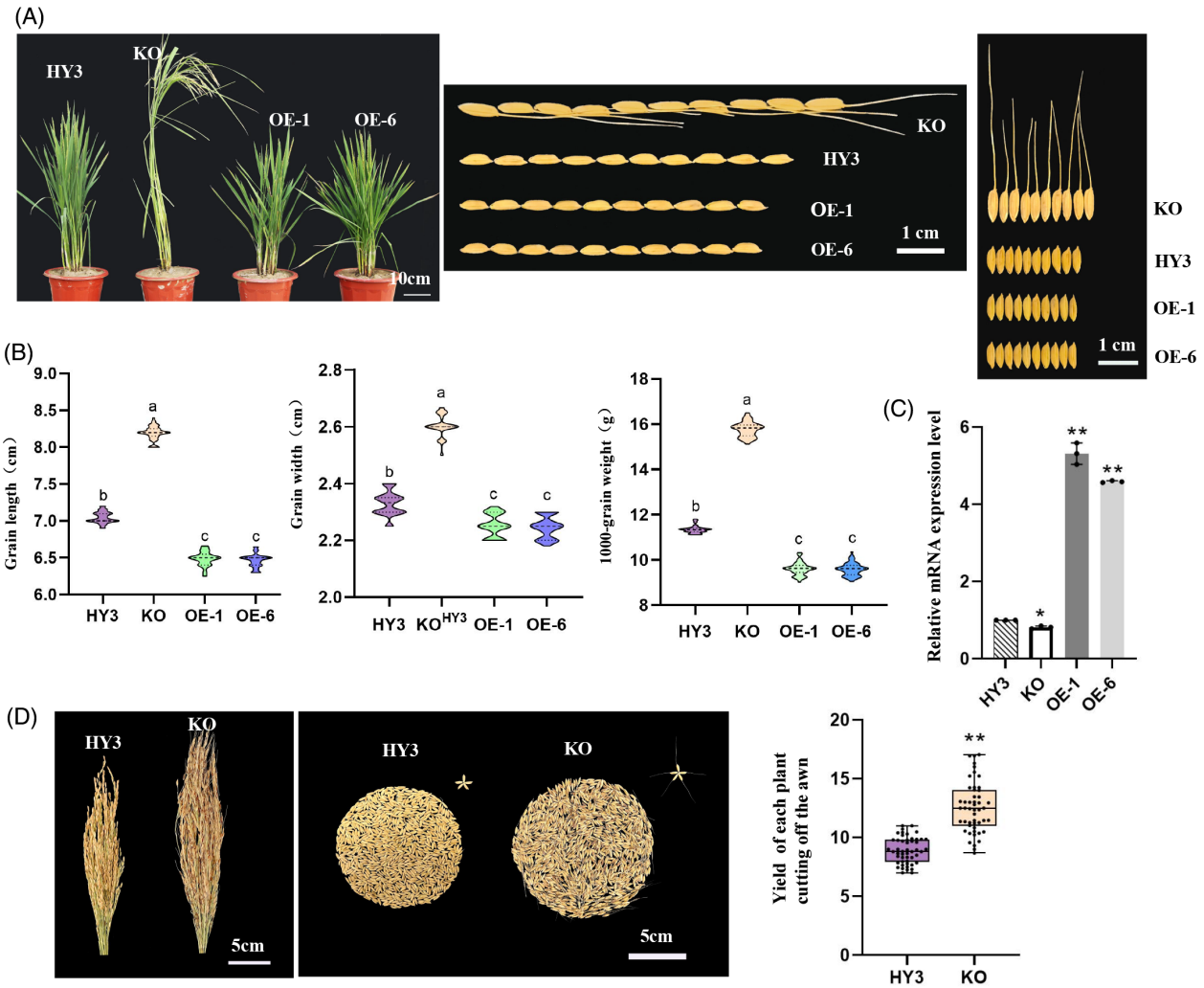


Figure 2. Phenotype analysis and mRNA expression analysis of *OsGSW3.2* in the transformed plants.
(A) Plant type, grain length, and grain width assays for the KO, OE-1, and OE-6 T_3 transgenic lines and wild-type HY3.
(B) Differential analysis of grain length, grain width, and 1000-grain weight in the T_3 KO lines and T_3 OE lines ($n = 50$). Violin plots were constructed in GraphPad. Different letters indicate statistically significant differences between groups ($P < 0.01$), as determined by Tukey's honest difference test. The transverse dotted line of each violin shows the quartile and median of each haplotype.
(C) The mRNA expression levels of *OsGSW3.2* in the T_3 KO lines and T_2 OE lines ($n = 3$).
(D) Yield analysis for each plant in the T_3 KO line. The data points ($n = 50$) are presented in box plots. The data are presented as the means ± SDs, and Student's *t* test was used to determine the *P* values. $*P < 0.05$, $**P < 0.01$.

which were downregulated (Figure 4A; Table S5). At the 50% FLG stage, 4349 DEGs were detected between the HY3 and KO lines, 1927 of which were upregulated and 2422 of which were downregulated (Figure 4A; Table S6). With hull development, the number of DEGs gradually increased; in total, 1214 common DEGs were detected at the two stages (Figure 4B). The mRNA expression levels of ten DEGs that were upregulated or downregulated were tested via RT–qPCR, and the results were consistent with those of the RNA-seq analysis (Figure 4C). These findings indicate that the transcriptomic data are reliable and can be used in subsequent research.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that at 25% FLG and 50% FLG, the pathways associated with the DEGs were enriched mainly in plant–pathogen interactions, plant hormone signal transduction, oxidative phosphorylation, starch and sucrose metabolism, and the MAPK signaling pathway–plant, and so on (Figure 4D). The phytohormone signaling pathway and the plant MAPK signaling pathway are the important pathways affecting grain size in rice. Therefore, *OsGSW3.2* may influence rice grain size by participating in the phytohormone signaling pathway and the MAPK signaling pathway.

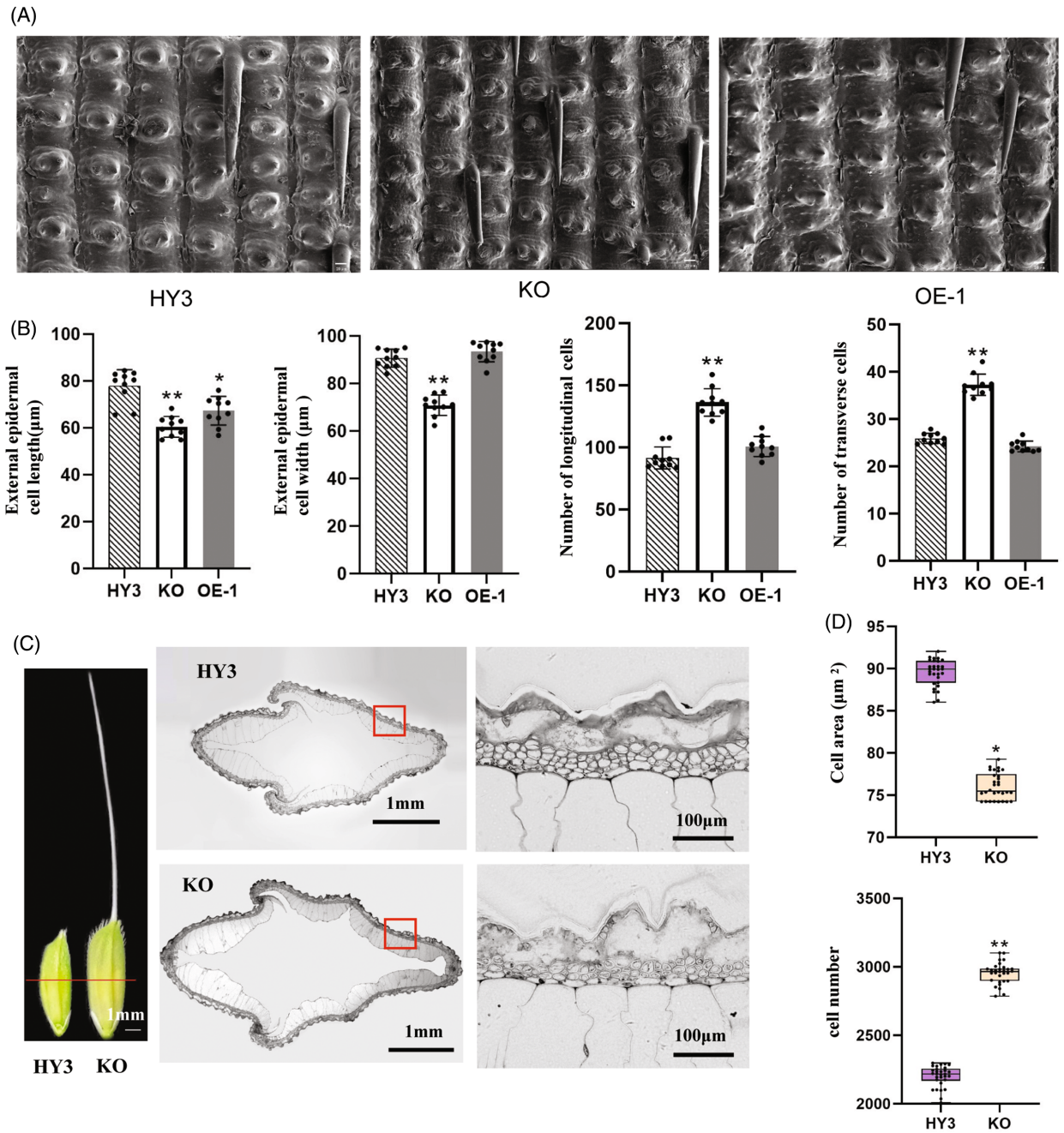


Figure 3. Cytological analysis of the glumes of the KO and OE-1 lines.
(A) Scanning electron microscopy observation of the central region of the grain outer surfaces for HY3, KO, and OE-1. The scale bars represent 20 μm.
(B) Significant difference analysis of the cell length and width of the external epidermis, longitudinal cell numbers, and transverse cell numbers of the spikelet glume between the wild-type HY3, KO, and OE-1 lines. The data points (n = 10) are presented in box plots; two grains of each sample were analyzed, and five photos were taken for each grain. The data are presented as the means ± SDs, and Student's t test was used to determine the P values. *P < 0.05, **P < 0.01.
(C) Optical microscopy observation of the grain outer surfaces of the HY3 and KO lines performed via plastic semithin section technology. The regions marked with a red box are enlarged (middle panel).
(D) Significant differences in the cell area and number of epidermal cells in the lemma were detected between the HY3 and KO lines. A total of 30 grains from each sample were cut into semithin sections, and three views of each grain's central-part section were analyzed. The data points (n = 30) are presented in box plots. The data are presented as the means ± SDs, and Student's t test was used to determine the P values. *P < 0.05, **P < 0.01.

There were many more DEGs detected at the 50% FLG stage than at the 25% FLG stage; therefore, the DEGs at the 50% FLG stage were subjected to in-depth analysis. Among the DEGs at the 50% FLG stage, some participated in grain size and awn development, and some participated in chlorophyll synthesis or degradation. For example, eight DEGs

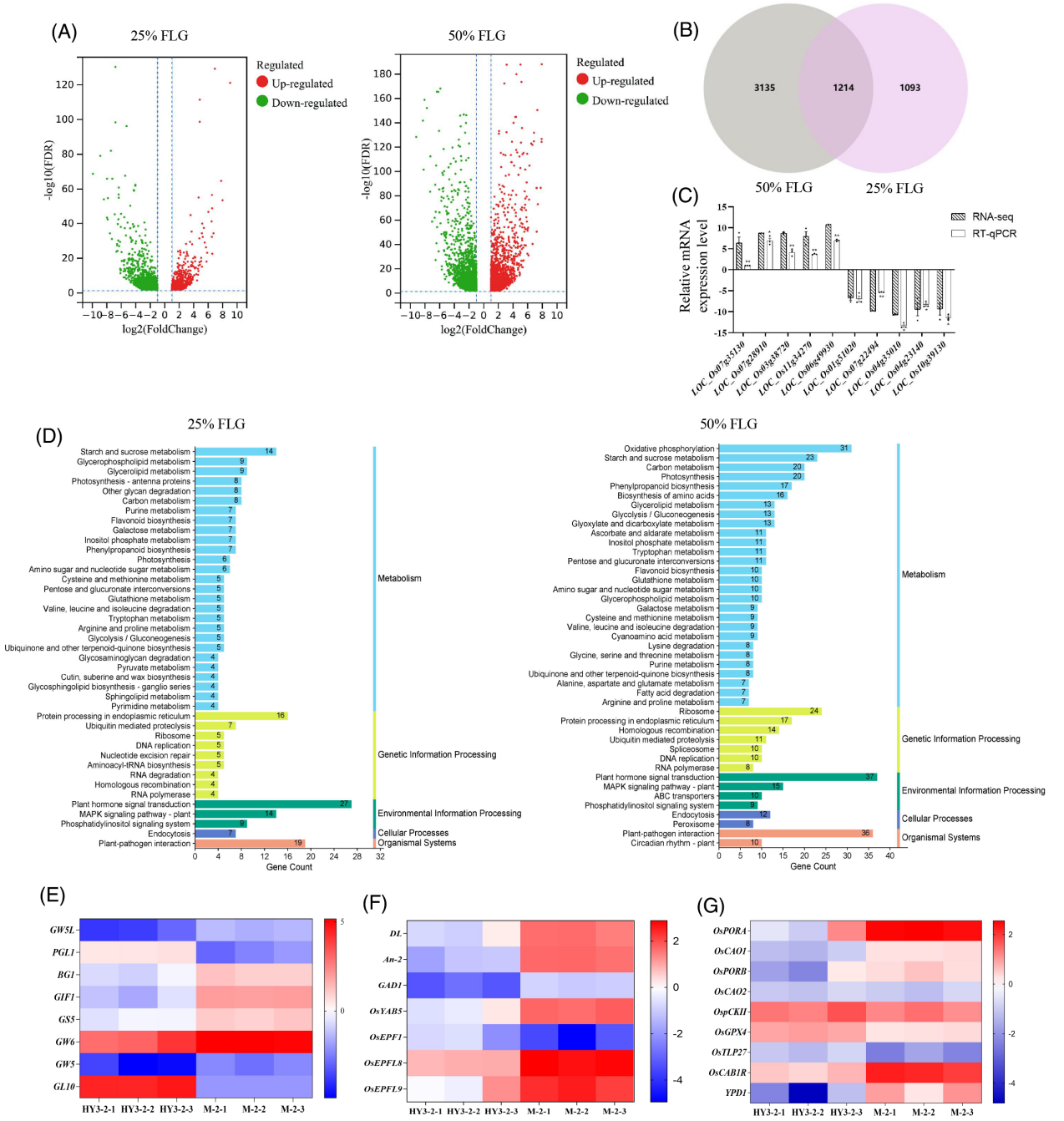


Figure 4. DEG analysis of the different developmental hulls of the KO and wild-type HY3 lines.
(A) Volcano plot of the differentially expressed genes (DEGs). From left to right, 25% full-length hulls of grains (FLG) and 50% FLG are shown; red dots represent upregulated genes, and green dots represent downregulated genes.
(B) Venn diagram of DEGs in different comparison groups.
(C) RT–qPCR analysis of ten DEGs whose expression was upregulated or downregulated ($n = 3$). The data are presented as the means ± SDs, and Student's t test was used to determine the P values. $^{*}P < 0.05$, $^{**}P < 0.01$.
(D) KEGG pathway enrichment analysis of DEGs for the 25% and 50% FLG of the HY3 and KO lines, respectively.
(E) Heatmap of DEGs related to grain length for the 50% FLG of the HY3 and KO lines.
(F) Heatmap of DEGs related to chlorophyll synthesis and degradation for the 50% FLGs of the HY3 and KO lines.
(G) Heatmap of DEGs related to awn development for the 50% FLG of the HY3 and KO lines. "M" indicates KO lines; "2-1, 2-2, 2-3" indicate the first replicate material, the second replicate material, and the third replicate material at 50% FLG, respectively; horizontal and vertical coordinates indicate the sample; and the number in the box represents the correlation coefficient of the two samples, while the darker the box is, the greater the correlation is.

were found to influence grain size: *GW5L*, *PGL1*, *BG1*, *GIF1*, *GS5*, *GW6*, *GW5*, and *GL10* (Figure 4E). Seven DEGs were found to influence awn development: *DL*, *An-2*, *GAD1*, *OsYAB5*, *OsEPF1*, *OsEPFL8*, and *OsEPFL9* (Figure 4F). Nine DEGs were found to participate in chlorophyll synthesis and degradation: *OsPORA*, *OsCAO1*, *OsPORB*, *OsCAO2*, *OspCKII*, *OsGPX4*, *OsTLP27*, *OsCAB1R*, and *YPD1* (Figure 4G). These results suggest that *OsGSW3.2* is an important gene that affects multiple agronomic traits in rice by upregulating or downregulating the mRNA expression levels of genes related to agronomic traits.

OsGSW3.2 influences the grain-filling rate

Grain development is closely related to hull size and the grain-filling rate. To explore the influence of *OsGSW3.2* on the grain-filling rate, the grain fresh weight and dry weight at different developmental stages were investigated for the KO, OE-1, and wild-type HY3 lines (Figure 5A). The fresh weight of the grains of the KO lines increased rapidly from 3 to 5 days after flowering (DAF) and peaked at 15 DAF. However, the grain fresh weights of the wild-type HY3 and OE-1 lines reached a maximum at 20 DAF (Figure 5B). Compared with the fresh weight of the HY3 grains, the fresh weight of the grains from the KO lines reached a maximum earlier, and the value was much greater, whereas the maximum of the OE-1 lines was later, and the value was slightly lower. The grain dry weight of the KO lines gradually increased during grain development, peaked at 15 DAF, and then remained stable. Similarly, the trends in the variation in the grain dry weight of the HY3 and OE-1 lines were basically consistent with those of the KO lines but peaked slightly later, similar to the changes in fresh weight (Figure 5C). Thus, *OsGSW3.2* influenced the grain-filling rate, and the significant increase in grain weight in the KO lines resulted in increases in both hull size and the rate of grain filling.

OsGSW3.2 is involved in BR signal transduction

The transcriptomic analysis revealed that the pathway enriched in the 27 DEGs at the 25% FLG stage and the 37 DEGs at the 50% FLG stage was plant hormone signal transduction (Figure 4D). Among the DEGs, some were related to brassinosteroid (BR) biosynthesis, such as *D2* and *Det2*, which have been reported in *Arabidopsis* (Figure S1). To verify the reaction of *OsGSW3.2* to BR, KO lines and the corresponding wild-type HY3 were used to investigate the phenotype under different concentrations of exogenous BL. Laminar inclination experiments revealed that the seedlings of the KO lines were significantly more sensitive to the exogenous brassinolide treatment than those of the wild-type HY3 plants (Figure 5D). With increasing concentration, the leaf angles of the KO lines and wild-type plants gradually increased (Figure 5E). The relative growth rate of the leaf angle of the KO lines to that of the wild-type HY3 plants first decreased, followed by a slight increase and then a rapid increase, reaching a peak of 86.02% at 500 nM, and subsequently dramatically decreased (Figure 5F). Therefore, 500 nM was used as the concentration for growth reversal under the exogenous BL treatment. Similarly, the mRNA expression levels of *OsGSW3.2* and ten BR-related signaling DEGs were studied further at 500 nM in the KO and HY3 lines, and almost all of these genes presented greater mRNA transcriptional accumulation in the KO lines than in the wild-type HY3 (Figure 5G). These results indicate that *OsGSW3.2* is involved in BR signal transduction to regulate plant development.

OsGSW3.2 interacts with OsGSK4 in yeast and plant cells

To elucidate the potential mechanism by which *OsGSW3.2* affects multiple agronomic traits, a yeast two-hybrid assay was performed to identify interacting proteins. The full-length cDNA of *OsGSW3.2* was cloned and inserted into the pGBKT7 vector to generate the pGBKT7-OsGSW3.2 fusion protein. Autotranscriptional activation was not detected with the full-length protein of OsGSW3.2. In addition, 21 genes related to grain size, BR signaling, chlorophyll synthesis and degradation, such as *GW2*, *YELLOW-GREEN LEAF 22* (*OsYGL22*), *GSK3/SHAGGY-LIKE KINASE1* (*OsGSK1*), and *OsGSK4*, were subsequently cloned and inserted into the pGADT7 vector. Using the point-to-point identification method of the two-yeast assay, pGBKT7-OsGSW3.2 was used as bait to search for the target protein. Three genes were identified as interacting with OsGSW3.2: OsYGL22, SHIKIMATE KINASE-LIKE 2 (OsSKL2), and HEAT SHOCK PROTEIN 24.1 (OsHSP24.1) (Figure 6A). *OsGSK4* was cloned and inserted into the pGBKT7 vector to generate the pGBKT7-OsGSK4 fusion protein, and *OsGSW3.2* was cloned and inserted into the pGADT7 vector to generate the pGADT7-OsGSW3.2 fusion protein. In the pGBKT7-OsGSK4 fusion protein, autotranscriptional activation was not detected. To our surprise, an interaction between OsGSW3.2 and OsGSK4 was detected (Figure 6A). Among the four interacting genes, OsGSK4 was related to BR, and *OsGSW3.2* was also verified to be involved in BR signal transduction. The interaction between OsGSK4 and OsGSW3.2 was further studied.

Bimolecular fluorescence complementation (BiFC) analysis was used to determine whether OsGSW3.2 interacted with OsGSK4. OsGSW3.2 was inserted into the vector pUC-SPYNE to construct the OsGSW3.2-YFP^N fusion protein; similarly, OsGSK4 was inserted into the vector pUC-SPYCE to construct the OsGSK4-YFP^C fusion protein. The two fusion proteins were coexpressed in the epidermal cells of *N. benthamiana* leaves. A yellow fluorescent signal was detected in the Golgi body and ribosomes of epidermal cells in *N. benthamiana* leaves (Figure 6B). Moreover, we also examined the interaction between OsGSW3.2 and

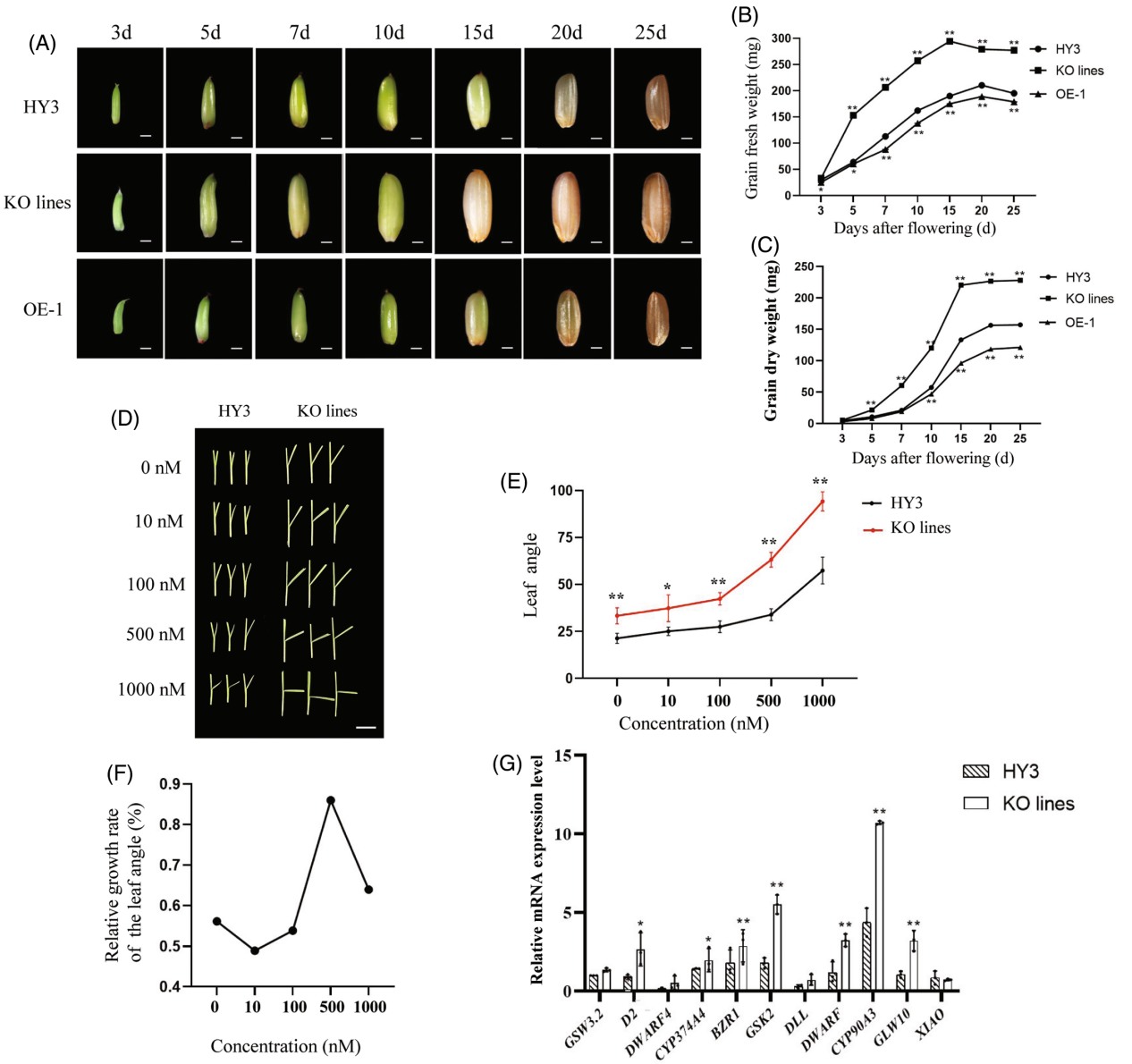


Figure 5. *OsGSW3.2* influenced the grain-filling rate and was involved in the BR signaling pathway.
(A) Grain phenotypes at different developmental stages of the HY3, KO, and OE lines. The scale bars represent 1 mm.
(B) Time course of grain fresh weight in the HY3, KO, and OE lines (n = 100 grains for each data point).
(C) Time course of grain dry weight in the HY3, KO, and OE lines (n = 100 grains for each data point).
(D) Phenotypes of the KO lines and HY3 plants treated with different concentrations of exogenous BL (n = 50). The scale bar represents 1 cm.
(E) Leaf angle analysis of the KO and HY3 lines under different concentrations of exogenous BL (n = 50).
(F) Relative growth rate of the leaf angle of the KO lines to that of the wild-type HY3 under different BL (n = 30).
(G) Relative mRNA expression levels of BR signaling-related genes in response to 500 nM BL, as determined by RT–qPCR in the KO lines (n = 3). The data are presented as the means ± SDs. Student's t test was used to determine the P values. *P < 0.05, **P < 0.01.

OsGSK4 via a split firefly luciferase complementation (LAC) assay in *N. benthamiana* leaf cells. The combination of OsGSW3.2 and OsGSK4 reconstituted functional LUC activity (Figure 6C). The interaction between OsGSW3.2 and OsGSK4 was also confirmed by a pull-down assay. The glutathione S-transferase (GST)-OsGSK4 and His-OsGSW3 fusion proteins were expressed in the *Escherichia coli* BL21 strain and purified by affinity chromatography. GST-OsGSK4 was able to pull down His-OsGSW3.2 but not GST, which suggests that OsGSK4 interacts directly with OsGSW3.2 (Figure 6D). Compared with the wild-type HY3, the mRNA expression of *OsGSK4* was significantly lower in the panicles throughout all the developmental stages in the KO lines (Figure 6E).

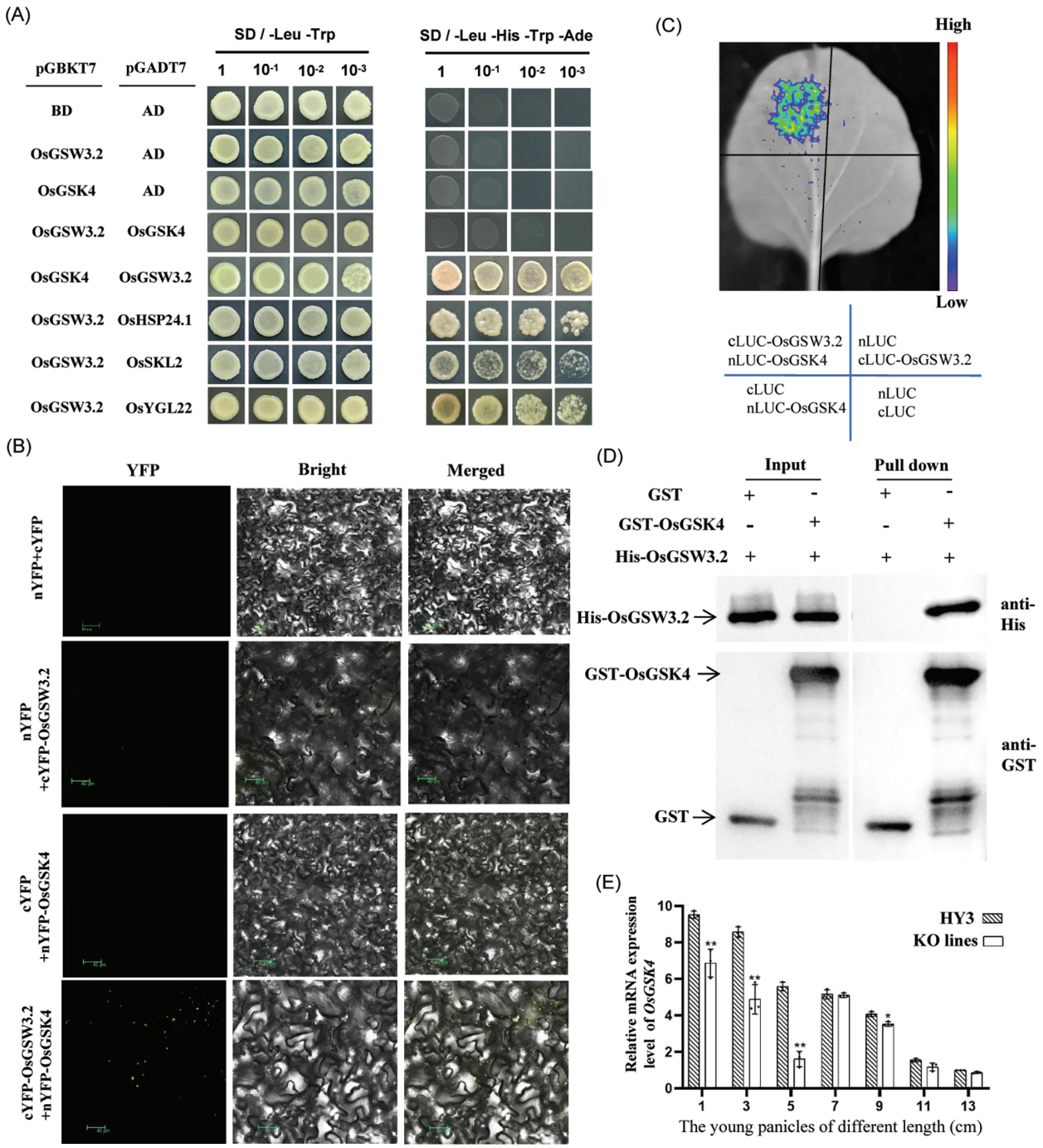


Figure 6. OsGSW3.2 interacted with OsGSK4 and the mRNA expression level of *OsGSK4* in the KO lines.
(A) GAL4-based yeast two-hybrid (Y2H) assay showing the interactions between OsGSW3.2 and OsGSK4, OsHSP24.1, OsSKL2, and OsYGL22. AD, activation domain; BD, binding domain; SD, synthetic dropout. The gradients indicate 10-fold serial dilutions.
(B) BiFC analysis showing the interaction between OsGSW3.2 and OsGSK4 in the epidermal cells of *Nicotiana benthamiana* leaves. The scale bars represent 40 μm.
(C) Split luciferase complementation indicates an in vivo interaction between OsGSW3.2 and OsGSK4 in *N. benthamiana* leaves.
(D) Pull-down assay showing the interaction between OsGSW3.2 and OsGSK4.
(E) The mRNA expression level of *OsGSK4* in the young panicles of different developmental stages for the HY3 and KO lines. The numbers of 1 to 13 on the abscissa axis represent the lengths of the young panicles, and the unit is centimeters (cm). The data are presented as the means ± SDs, and Student's *t* test was used to determine the *P* values. $*P < 0.05$, $**P < 0.01$.

These results further confirmed that OsGSW3.2 interacts with OsGSK4 in vivo and in vitro. *OsGSK4* plays an important role in regulating BR signaling, and the *gsk2,4* double mutation negatively regulates rice grain size (Liu et al., 2021). OsGSW3.2 regulates grain size and weight by interacting with OsGSK4, which is related to the BR signaling pathway.

Natural variation at the *OsGSW3.2* locus affects grain size and weight

The genomes of *OsGSW3.2* for the two parents (KJ01 and HY3) were sequenced, and seven SNPs were generated in the coding region. To investigate the possible grain size and weight effects of natural variation in *OsGSW3.2*, the sequences of 2741 accessions of cultivated rice (3K rice genomes) from 89 countries were analyzed (https://www.rmbreeding.cn/Index/). Using the seven SNPs in the coding region, the accessions were divided into five haplotypes (Figure 7A). All the haplotypes except C-Hap2 were found in *indica* accessions, but there were far more accessions (1551) with C-Hap1 than with the other haplotypes. Three haplotypes (C-Hap1, C-Hap2, and C-Hap3) were found among the *japonica* accessions, whereas there were far more accessions (673) harboring C-Hap3 than those harboring C-Hap1 and C-Hap2. Three (C-Hap1, C-Hap3, and C-Hap4) were found among the Asu accessions, and the number of accessions (147) harboring C-Hap4 was the greatest. Among the accessions, C-Hap1 was present mainly in *indica* (96.16%), and only 0.43% was present in *japonica*; C-Hap2 was present mainly in *japonica* cultivars (96.23%), and C-Hap4 was not present in *japonica*, with C-Hap4 present mainly in Asu (57.65%) and 33.33% in *indica*; C-Hap3 was present in all the cultivars, and mainly in *japonica* (88.20%), and only 2.10% in *indica* (Figure 7A). These findings indicate that the *OsGSW3.2* gene may be involved in *indica*–*japonica* subspecies differentiation.

The variable bases of *OsGSW3.2* in HY3 were classified as C-Hap1, and those SNPs in KJ01 were classified as C-Hap2. Compared with those in C-Hap1, only one SNP (+668) was different in C-Hap5. At position +668, the degenerate base R (A/G) encoded Arg^{165}/Gly^{165} in C-Hap5, which encoded Arg^{165} in C-Hap1. Compared with those with C-Hap1, the accessions with C-Hap5 generated significantly smaller grains. Only one SNP (+740) was different between C-Hap3 and C-Hap2, which caused amino acid variation. At position +740, Base C encoded Arg^{189} in C-Hap3, and the substitution base T encoded Trp^{189} in C-Hap2. Species with C-Hap3 had the second-longest grains (8.61 mm), those with C-Hap2 (KJ01) had the shortest grains (7.80 mm). But the species with C-Hap3 had a narrower grain width (3.24 mm) than those of harboring C-Hap2 (3.41 mm), and their grain weight was not significantly different (Figure 7B).

To further explore which amino acid variation influences grain size and weight by interacting with OsGSK4, the coding sequence of *OsGSW3.2* was divided into three fragments according to the position of the three non-synonymous SNPs, which were subsequently cloned and inserted into the pGADT7 vector to generate the fusion proteins pGADT7-OsGSW3.2^{N} (N-terminal, including the non-synonymous SNP of the +668 position), pGADT7-OsGSW3.2^{M} (middle section, including the non-synonymous SNP of the +740 position) and pGADT7-OsGSW3.2^{C} (C-terminal, including the non-synonymous SNP of the +812 position) (Figure 7C). The interactions of the three fusion proteins of OsGSW3.2^{N}, OsGSW3.2,^{M} and OsGSW3.2^{C} with OsGSK4 were detected via a yeast two-hybrid assay. The results confirmed the interaction of only OsGSW3.2^{N} with OsGSK4 (Figure 7C). These results suggest that OsGSW3.2 may influence grain size and weight via interaction with OsGSW3.2^{N} and that the non-synonymous SNP at position +668 was probably very critical for grain development.

Transcriptional efficiency influences grain size and weight

The mRNA expression level of *OsGSW3.2* was much greater in HY3 than in KJ01, especially in the panicles. Notably, greater accumulation of the *OsGSW3.2* mRNA resulted in smaller grains in the OE lines than in the KO lines. We speculate that the transcriptional level of *OsGSW3.2* may influence grain size and weight. Transcription efficiency is closely related to the promoter, and the impact of differential SNPs of *OsGSW3.2* in the promoter region on grain size was analyzed via 2785 accessions of cultivated rice sequences from the 3K rice genome database. The promoter region of *OsGSW3.2* in the HY3 and KJ01 lines generates eight SNPs in the 1000 bp region upstream of the initiation codon. The eight SNPs were divided into three haplotypes; the variable bases of *OsGSW3.2* in HY3 were classified as P-Hap1, and those SNPs in KJ01 were classified as P-Hap2 (Figure 7D). The grain size and weight presented a significantly different outcome between P-Hap1 and P-Hap2 (Figure 7E). Only two SNPs differed between P-Hap3 and P-Hap2, and the P-Hap3 accessions presented reduced grain size and weight.

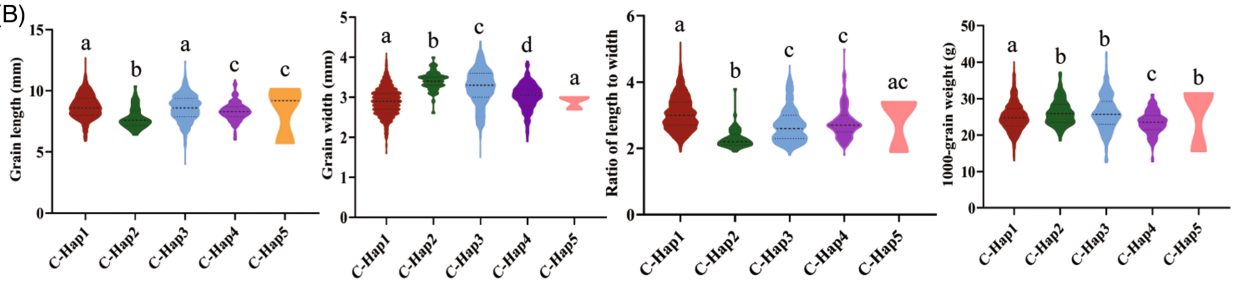
Subsequently, the haplotype combinations of the coding region and promoter region were further analyzed. It was worth noting that the accessions harboring C-Hap1 and C-Hap5 in the coding region had the common promoter haplotype P-Hap1, but there was only one SNP at position +668 and presented an obvious difference in grain length and weight (Figure 7F). Similarly, the accessions harboring C-Hap2 and C-Hap3 in the coding region also had the common promoter haplotype P-Hap2, and there was only one SNP at position +740, which had obvious differential grain length and grain width, too (Figure 7F). We speculated that the SNPs at positions +668 and +740 were

all very important to grain development; perhaps they influenced grain development in different pathways, and the SNP at position +668 impacted grain size and weight by interacting with OsGSK4. The accessions harboring C-Hap3 and C-Hap4 in the coding region had only two SNPs differences at positions +295 and +757, which were silent

(A)

Haplotypes	+295	+511	+668	+740	+757	+812	+824	Asu	Bas	*japonica*	*indica*	admix	Num.	GL(mm)	GW(mm)	GL/GW	TGW(g)
C-Hap1(HY3)	C	T	A	C	G	T	T	19	2	7	1551	34	1613	8.70	2.89	3.06	24.99
C-Hap2(KJ01)	T	C	G	T	A	C	C	0	0	102	0	4	106	7.80	3.41	2.30	26.25
C-Hap3	T	C	G	C	A	C	C	11	43	673	16	20	763	8.61	3.24	2.71	26.14
C-Hap4	C	C	G	C	G	C	C	147	15	0	85	8	255	8.30	3.02	2.80	23.42
C-Hap5	C	T	R	C	G	T	T	0	0	0	4	0	4	8.37	2.90	2.90	26.13

(B)

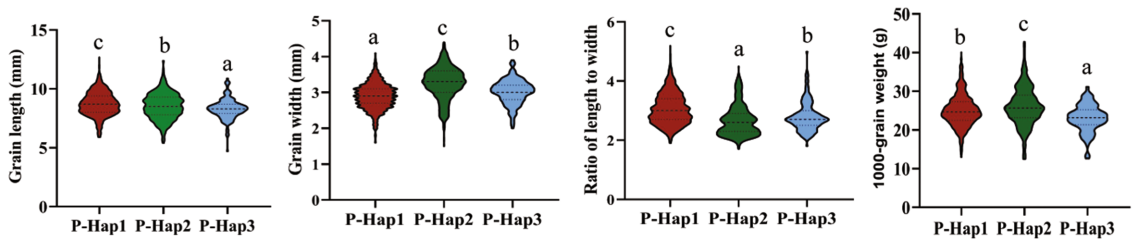


(C)

(D)

Haplotypes	-193	-438	-525	-719	-747	-761	-893	-905	Asu	Bas	*Japonica*	*indica*	admix	Num.	GL(mm)	GW(mm)	GL/GW	TGW(g)
P-Hap1(HY3)	A	T	G	C	T	A	A	C	20	1	7	1571	36	1635	8.72	2.89	3.06	25.04
P-Hap2(KJ01)	T	G	A	G	C	G	G	T	12	46	785	18	24	885	8.53	3.26	2.67	26.11
P-Hap3	T	G	A	G	T	G	G	C	156	19	0	82	8	265	8.31	3.02	2.8	23.09

(E)



(F)

Haplotypes in the promoter region	Haplotypes in the coding region	+295	+511	+668	+740	+757	+812	+824	Asu	Bas	*japonica*	*indica*	admix	Num.	GL(mm)	GW(mm)	GL/GW	TGW(g)
P-Hap1(HY3)	C-Hap1(HY3)	C	T	A	C	G	T	T	18	1	7	1504	33	1563	8.70	2.89	3.06	25.06
	C-Hap5	C	T	R	C	G	T	T	0	0	0	4	0	4	8.37	2.90	2.90	26.13
P-Hap2(KJ01)	C-Hap2(KJ01)	T	C	G	T	A	C	C	0	0	98	0	4	102	7.79	3.40	2.30	26.23
	C-Hap3	T	C	G	C	A	C	C	11	14	640	15	8	688	8.62	3.24	2.72	26.14
P-Hap3	C-Hap4	C	C	G	C	G	C	C	144	14	0	78	8	244	8.31	3.03	2.79	23.34

Figure 7. Haplotype analysis of the SNPs in the coding region and promoter region of *OsGSW3.2*.
(A) The haplotypes for the SNPs in the coding region of *OsGSW3.2*, where R represents A/G.
(B) Significant difference analysis of the grain length (GL), grain width (GW), ratio of grain length to width (GL/GW), and thousand-grain weight (TGW) for the six haplotypes. Bas, Basmati; Num., number of total accessions in each category; the SNPs at the position in purple bold font resulted in amino acid variation. A violin plot was constructed in GraphPad. Different letters indicate statistically significant differences between groups ($P < 0.01$), as determined by Tukey's honest difference test. The transverse dotted line of each violin shows the quartile and median of each haplotype.
(C) The analysis of the impact of non-synonymous SNPs on grain size. OsGSW3.2 was divided into three fragments according to the position of the three non-synonymous SNPs, and each fragment was detected for the interaction with OsGSK4 via a yeast two-hybrid.
(D) The haplotypes for the SNPs in the promoter region of *OsGSW3.2*.
(E) Significant difference analysis of GL, GW, GL/GW, and TGW for the three haplotypes. Different letters indicate statistically significant differences between groups ($P < 0.05$), as determined by Tukey's honest difference test. The transverse dotted line of each violin shows the quartile and median of each haplotype.
(F) The analysis of haplotype combinations for the SNPs in the promoter and coding region.

mutations and had not changed the amino acid in the translation process, but they generated obvious differential grain size and weight (Figure 7F). Further analysis revealed that their promoter haplotypes were respectively P-Hap2 and P-Hap3, which had two SNPs differences and generated significant differential grain size and weight (Figure 7E). Therefore, we speculated that the difference in grain size and weight between the accessions harboring C-Hap3 and C-Hap4 was probably caused by the SNPs of promoter of *OsGSW3.2*. The SNPs of *OsGSW3.2* in the promoter region may affect grain size and weight by influencing transcription efficiency. The hereditary basis of these cultivated varieties was very complex. Therefore, those results should be further verified experimentally.

DISCUSSION

The wild-type allele *OsGSW3.2* is an ancient gene

Oryza rufipogon and *Oryza barthii* are the direct ancestor species of Asian cultivated rice and African cultivated rice, respectively. Grain shape and shattering are considered the main traits distinguishing wild rice from cultivated rice, so grain size, non-shattering, and awnlessness are also called domestication traits (Hasan et al., 2023). Rice domestication involves the evolutionary force of a series of critical domestication alleles, including gene flow, artificial selection, and natural selection (Sang & Ge, 2013). During domestication, grain size, which is also a significant trait for studying rice evolution, was selected for breeding. Current cultivated varieties were generated based on the basis of the selected domestication alleles. To date, an increasing number of domestication genes have been cloned. *GRAIN SIZE ON CHROMOSOME 3* (*GS3*) and *QTL FOR SEED WIDTH ON CHROMOSOME 5* (*qSW5*) were reported to increase grain length and width for the single loss function of each gene selected during rice domestication (Fan et al., 2006; Shomura et al., 2008). The wild-type *An-1*, *LABA1*, and *REGULATOR OF AWN ELONGATION 2* (*RAE2*) alleles, which regulate long-term development, have been cloned (Bessho-Uehara et al., 2016; Hua et al., 2015; Luo et al., 2013). *GRAIN LENGTH AND AWN DEVELOPMENT* (*GLA*), the allele gene of *GAD1/RAE2*, encodes one member of the *epidermal patterning factor-like protein* (EFPL) family. InDel variation in the promoter region of *GLA* increases grain length by promoting the transcription of *GLA*, and the absence of InDel in the coding region of *GLA* results in short awn or no awn phenotypes (Zhang et al., 2019). Wang et al. (2019) reported that *GRANIN LENGTH AND AWN 1* (*Gla1*) caused significant increases in grain length and weight and was associated with long awns. GLA1 negatively regulates grain size via the dephosphorylation of OsMAPK6. *REGULATOR OF AWN ELONGATION 3* (*RAE3*) encodes an E3 ubiquitin ligase and was found to be responsible for the awnless phenotype during African rice domestication (Bessho-Uehara et al., 2023).

In our study, *OsGSW3.2*, which was derived from the wild-type rice mutant HY3, was knocked out in the HY3 background, and the KO lines presented long awns, larger grains, and yellow–green leaves. The wild rice allele *OsGSW3.2* influences multiple agronomic traits, including grain and awn development. OsGSW3.2 was located in the Golgi body and ribosome of the rice cytoplasm (Figure 1C, D). The Golgi body is related to the formation of cellular secretions and works to process and transport proteins from the endoplasmic reticulum. The ribosome is an important organelle for protein translation. These results suggested that *OsGSW3.2* is an important gene related to rice growth. A yeast two-hybrid assay revealed that OsGSW3.2 interacted with many different functional proteins, such as OsYGL22, OsSKL2, OsGSK4, and OsHSP24.1. OsGSW3.2 may be involved in multiple signaling pathways that influence different agronomic traits. Haplotype analysis revealed that the SNPs of *OsGSW3.2* were divided into six haplotypes in the rice cultivar. Among them, the wild rice allele *OsGSW3.2* (C-Hap1) was present in 96.16% of the *indica* accessions, and C-Hap3 was present in 88.20% of the *japonica* accessions. These results indicated that *OsGSW3.2* was an ancient gene that may be involved in *indica–japonica* differentiation in rice.

OsGSW3.2 negatively regulates grain size and influences rice yield

In recent years, many genes related to grain size have been cloned, and their molecular functions have been further

studied. However, these plants are seldom derived from wild rice or the simultaneous regulation of multiple agricultural traits. In this study, we identified a novel wild rice allele, *OsGSW3.2*, which negatively regulates grain size by suppressing cell division. *OsGSW3.2* has pleiotropic effects on grain size, awn development, chlorophyll synthesis, and other processes. Although the tiller number of the KO lines decreased, their yield increased with increasing length and number of grains per panicle. Compared with that of wild-type HY3, the yield of the KO lines increased by 48.31%. RNA-seq revealed that eight genes related to grain size, including *GW5L*, *PGL1*, *BG1*, *GIF1*, *GS5*, *GW6*, *GW5*, and *GL10*, were upregulated or downregulated. *OsGSW3.2* is an important gene for understanding rice domestication and improving rice yield. Awns can also influence the entry angle of seeds into the soil to promote seed dispersal and germination and further improve plant survival (Jin et al., 2023). *OsGSW3.2* will be beneficial for improving seeds to meet the mechanical live-stream breeding requirements.

The quantitative trait locus *RICE BIG GRAIN1* (*qRBG1*) from the single-segment substitution line Z499, which has natural promoter variation, reduces *qRBG1* expression and enhances rice yield (Zhang et al., 2024). In our studies, in the KO lines, OE lines, and parents of HY3 and KJ01, the higher the mRNA expression level of *OsGSW3.2*, the smaller the grain. Transcription efficiency is closely related to the promoter. Haplotype analysis revealed that the differential SNPs at positions −747 and −905 in the promoter region of *OsGSW3.2* reduce grain size and weight. The differential SNPs in the promoter region affect the mRNA expression level of *OsGSW3.2*. In addition, the nonsynonymous SNPs at the +668 and +740 positions of the coding region of *OsGSW3.2* influence grain size and weight, and OsGSW3.2^{N}, with various amino acids at the +668 position, can interact with OsGSK4. These results showed that *OsGSW3.2* may influence grain size and weight in rice via differential SNPs in the coding region that cooperate with the mRNA expression level.

OsGSW3.2 participated in regulating leaf color

When chlorophyll metabolism cannot maintain proper function, rice plants exhibit a wide range of leaf color changes, which also affect other growth phenotypes, such as plant height, fertility, tiller number, and yield (Li et al., 2021; Lisiewska et al., 2006; Niu et al., 2022; Ramamoorthy et al., 2018; Yu et al., 2021). An 18-bp insertion in *ALBINO LENTHAL14* (*AL14*) occurred in front of the termination codon, resulting in a change in the polypeptide chain. At the seedling stage, the *al14* mutant presented albinotic leaves and turned green at the three-leaf stage, which also affects heading date and plant height, with no significant changes in other agronomic traits (Hu et al., 2021). A single nucleotide substitution (G to T) in *WHITE GREEN LEAF 2* (*WGL2*) changed the amino acid sequence from glycine to valine, and the resulting mutant *wgl2* exhibited an albino phenotype from the germination to the three-leaf stage and gradually transitioned to green during later development (Qiu et al., 2018). *OsYGL22* may be involved in the early development of chloroplasts in rice, and the *ygl22* knockout mutant presented a yellow–green leaf phenotype but did not significantly affect plant height, the number of panicles per plant, or grain yield per plant (Zhu et al., 2021).

Like those of *WGL2*, the *KO* lines of *OsGSW3.2* presented an albino phenotype from the germination stage to the two-leaf stage, and the plants gradually turned yellow–green during late development. According to our transcriptomic analysis, nine upregulated or downregulated DEGs were found to participate in chlorophyll synthesis and degradation. A yeast two-hybrid assay revealed that OsGSW3.2 interacted with OsYGL22. OsGSW3.2 may regulate the chlorophyll content of leaves by interacting with OsYGL22, but the regulatory mechanism should be studied further. Leaf color can be used as a trait marker in hybrid rice production, and it is highly valuable to study the chlorophyll deficiency mechanism in detail.

OsGSW3.2 is involved in the BR signaling pathway in rice

BRs are classic plant-specific steroid phytohormones that control major aspects of plant growth and development in both dicots and monocots. In recent years, an increasing number of studies have revealed that BRs are involved in grain size and plant development in rice. *GRAIN LENGTH ON CHROMOSOME 3* (*qGL3*) encodes a protein phosphatase that contains Kelch-like repeat domains and suppresses BR signaling by dephosphorylating and stabilizing *Oryza sativa glycogen synthase kinase 3* (*OsGSK3*) (Gao et al., 2019). *SMALL GRAIN 2* (*SG2*) encodes a plant-specific protein with a ribonuclease H-like domain. SG2 is a component of the GSK2-related BR signaling response and regulates grain size by interacting with OsOFP19 (Huang et al., 2022). *OsBES1-4* plays a role in rice grain size development by binding to the BR response element in the promoter region of the *OsBZR1* gene and has the potential to be an important target in rice breeding programs (Cheng et al., 2023).

In our study, transcriptomic data revealed that some DEGs related to brassinosteroid biosynthesis were upregulated or downregulated in the KO lines (Figure S1). Compared with the wild-type HY3 plants, the KO lines presented taller plants and longer grains. Under exogenous BL treatment, the seedlings of the KO lines were more sensitive than those of the wild-type HY3. These results showed that *OsGSW3.2* was involved in the BR signaling pathway. Yeast two-hybrid, BiFC, LAC, and pull-down experiments verified that OsGSW3.2 interacted with OsGSK4. *OsGSK4* is related to BR signal transduction, and

the knockdown of *OsGSK4* by RNA interference (RNAi) enhances BR signaling (Tong et al., 2012). These results indicated that OsGSW3.2 regulated multiple rice traits, including grain size, via interactions with OsGSK4. The interaction mechanism by which OsGSW3.2 and OsGSK4 regulate grain size needs further study.

CONCLUSION

OsGSW3.2 is an ancient gene generated during rice domestication and has pleiotropic effects on grain size, awn development, and chlorophyll synthesis. OsGSW3.2 regulates grain size and weight by interacting with OsGSK4. After *OsGSW3.2* was knocked out via CRISPR/Cas9, all three editing types caused a code-shifting mutation in *OsGSW3.2*, which was associated with significant differences in agronomic traits, such as increased grain size, long awn, and low chlorophyll content. *OsGSW3.2*^{HY3} was overexpressed in the HY3 background, and the grain length and grain width of the OE mutants were shorter and narrower than those of the wild-type HY3. *OsGSW3.2* regulated grain size via the cell proliferation of spikelet glumes. Transcriptomic analysis revealed that the DEGs were enriched mainly in plant–pathogen interactions, plant hormone signal transduction, oxidative phosphorylation, starch and sucrose metabolism, and the plant MAPK signaling pathway, and so on. The results of lamina inclination experiments indicated that *OsGSW3.2* was involved in the BR signaling pathway. OsGSW3.2 interacts with multiple different functional proteins, such as OsGSK4, OsSKL2, OsHSP24.1, and OsYGL22. OsGSW3.2 influenced rice grain size by interacting with OsGSK4. Haplotype analysis revealed that *OsGSW3.2* influenced grain size and weight in rice via differential SNPs in the coding region that cooperate with the mRNA expression level. Our studies provide new insight into how *OsGSW3.2* regulates grain size and weight during rice domestication.

EXPERIMENTAL PROCEDURES

Plant materials

The wild rice (*O. rufipogon* Griff.) inbred line Huaye 3 (Yu et al., 2018), the cultivars *O. sativa* L. KJ01, and Nipponbare were used as the study materials. These materials were planted on the farm of South China Agricultural University from 2019 to 2024.

Agronomic traits and grain-filling analysis

Agronomic traits, including grain length, grain width, panicle length, and plant height, were calculated and measured according to Bai et al. (2023). All grains and panicles from each plant were used to measure the yield per plant and panicle length. After harvesting, approximately 300 fully filled grains per plant were used for measuring grain length, width, and weight, and all the samples were analyzed three times. The grains from the KO T_8 lines and the wild-type HY3 line were labeled at the time of flowering, after which the grains at 3, 5, 7, 10, 15, 20, and 25 days after flowering (DAF) were collected. The fresh weight and dry weight of 100 grains at each time point were analyzed.

RNA isolation and RT–qPCR analysis

Total RNA was extracted from different tissues of HY3, KJ01, and the transgenic lines via the TRIzol method (Yeasen), and first-strand cDNA synthesis was performed via a reverse transcription kit (Hifair III 1st Strand cDNA Synthesis SuperMix for qPCR [gDNA digester plus]). RT–qPCR was carried out according to the manufacturer's instructions. *OsActin 1* was used as an internal control. Three biological replicates and 3 technical replicates were included for each sample. The sequences of the primers used for RT–qPCR are listed in Table S7.

Vector construction and plant transformation

The CRISPR/Cas9 gene editing construct of *OsGSW3.2* was designed as previously described (Zeng et al., 2018). Two targets were designed for *OsGCA*^{HY3} via the CRISPR-GE website (http://skl.scau.edu.cn/). The knockout vector of *OsGSW3.2* was constructed according to Bai et al. (2023). The successfully constructed plasmid was subsequently introduced into *Agrobacterium tumefaciens* strain EHA105, which was subsequently reduced to HY3. The full-length coding sequence (CDS) of *OsGSW3.2* was subsequently cloned and inserted into the plant binary vector pCAMBIA1300S to generate the overexpression vector. The successfully generated plasmid was subsequently introduced into HY3 via *Agrobacterium*-mediated transformation. A 1000-bp promoter fragment of *OsGSW3.2* was cloned and inserted into the pCAMBIA13001 vector to generate the *OsGSW3.2-GUS* plasmid. The *Agrobacterium*-mediated method was used to transform the constructed plasmid into the rice variety Nipponbare. A 720-bp fragment of *OsGSW3.2* cDNA was amplified to generate the pBin19:eGFP-OsGSW3.2 and pOX:eGFP-OsGSW3.2 vectors.

GUS assays

Different tissues of the transgenic plants were dipped in GUS-staining solution (Leagene Biotechnology, β-Galactosidase Reporter Gene Staining Kit) for 24 h at 37°C, after which the stained tissues were dehydrated in 70% (*v*/*v*) ethanol to remove chlorophyll (Qi et al., 2012) and then observed under a stereoscope.

Rice glume plastic semithin slices

Before flowering, the spikelet shells of the HY3 and KO lines were collected and fixed in FAA fixative (5% formaldehyde, 6% glacial acetic acid, and 89% ethanol [70%]; *v*/*v*) for 48 h. Plastic semipackets of the rice glumes were subsequently prepared via a previously described method (Lu et al., 2020). Thirty spikelets from each sample were sliced into 5 μm thick sections and then stained and observed. The cell area and number of cells were determined via ImageJ software. The cell area of each cell derived from a transverse section at the central part of the spikelet was calculated via ImageJ software, and the average value of these cell areas was used as the single-cell area. The cross-sectional area of the whole rice glume was calculated via ImageJ and then divided by the average cell area, then obtaining the cell numbers of the transverse section.

SEM observation of rice glumes

The spikelet shells of the mature HY3, KO, and OE-1 lines were selected and dried completely, after which the samples were

processed and analyzed according to a previously described method (Liu et al., 2022; Wang et al., 2022). The cell length and width were determined via ImageJ software, and the average length and width of the cells were used as the longitudinal cell length and transverse cell width, respectively. The length and width of the rice grains were measured with a ruler, which were divided by the average length of the longitudinal cells and the average width of the transverse cells, respectively. The values obtained were used as the longitudinal cell numbers and the transverse cell numbers. All three grains of each material were observed and analyzed.

Subcellular localization

In accordance with a previously described protocol (Bai et al., 2023), the successfully constructed plasmids for pBin19-eGFP-OsGSW3.2, pBin-eGFP, and pBin19 were purified and injected into the lower epidermis of *N. benthamiana* leaves. After 1–2 days of incubation at room temperature, the lower epidermis of the injected *N. benthamiana* plants was removed and observed under a Leica SPE laser scanning confocal microscope (excitation spectrum: 488 nm; emission spectrum: 510 nm). The fusion protein pOX-GFP-OsGSW3.2 was transfected into rice protoplasts, and RPL27AB-mKATE and Golgi-mCherry were used as the markers according to a previously described method with minor modifications (Yoo et al., 2007). pOX-GFP alone was used as a control. The fluorescence was observed under a Nikon C2-ER laser scanning confocal microscope (excitation spectrum: 561 nm; emission spectrum: 580 nm).

RNA-seq analysis

The total RNA derived from 25% to 50% of the full-length glumes of the wild-type HY3 and KO lines was extracted and then sent to the BioMarker Biotechnology Company for RNA-seq analysis, with three biological replicates for each period of each material. The reads were aligned to the rice genome (MSU7.0) via TopHat. DEGs were identified based on $|\log2 \text{ (fold change)}| > 2$ and FDR < 0.01.

BL treatment-related lamina inclination assays

Lamina inclination assays after the BL treatment were performed as previously described with some modifications (Xie et al., 2023). Seeds of uniform size were selected from the wild-type HY3 and KO lines, treated with 75% alcohol (v/v) for 1–2 min, and rinsed with sterile water 3–4 times. These seeds were soaked in an incubator to promote germination. The lamina of 8-day-old plants was treated with 0, 0.01, 0.1, 0.5, or 1 μM BL. Images were captured 48 h after treatment, and the angles of lamina joint bending were measured via ImageJ software. Three biological replicates were used, each containing 30 plants.

Yeast two-hybrid analysis

The full-length coding regions of *OsGSW3.2*^{HY3} and *OsGSK4* were cloned and inserted into the pGBKT7 vector, which contains a binding domain (BD). Twenty-one genes (*GW2*, *YGL22*, *OsGSK4*, etc) with full-length coding regions were cloned and inserted into the pGADT7 vector, which contains an activating domain (AD). The interaction assay was performed according to the manufacturer's instructions (Clontech Yeast Protocols Handbook). Both the bait and prey constructs were cotransformed into the yeast strain AH109, which was subsequently grown on SD/−Leu/−Trp media and then further selected on SD/−Leu/−Trp/−His/−Ade media.

Bimolecular fluorescence complementation (BiFC) assays

The full-length coding sequence of *OsGSW3.2* without a stop codon was cloned and inserted into pUC-SPYNE vectors to construct OsGSW3.2-YFP^N. The full-length coding sequence of *OsGSK4* without a stop codon was cloned and inserted into pUC-SPYCE to construct OsGSK4-YFP^C. The resulting strains were subsequently transformed into *A. tumefaciens* strain EHA105 and coinjected into the epidermal cells of *N. benthamiana* leaves, as described previously (Zhang et al., 2018). Yellow fluorescence was observed with a confocal microscope 1–3 days after infiltration.

Luciferase complementation (LAC) assay

The full-length coding sequence of *OsGSW3.2* was cloned and inserted into the vector pCAMBIA1300-cLuc carrying the C-terminal half of luciferase to obtain the recombinant plasmid cLUC-OsGSW3.2, and the full-length coding sequence of OsGSK4 was cloned and inserted into the vector pCAMBIA1300-nLuc carrying the N-terminal half of luciferase to obtain the recombinant plasmid nLUC-OsGSK4. The two recombinant plasmids nLUC-OsGSK4 and cLUC-OsGSW3.2 were subsequently cotransformed into tobacco leaves by coupling Agrobacterium GV3101 strains. The leaves were sprayed with 1 mM D-luciferin potassium salt (Promega, P1043, USA) and observed with a CCD plant imaging apparatus. cLUC and nLUC were used alone as controls.

Pull-down assay

The full-length coding sequence of *OsGSW3.2* was inserted into the expression vector pET28a to obtain the His-OsGSW3.2 fusion protein. The coding region of *OsGSK4* was inserted into the expression vector pEGX4T-1 to obtain the GST-OsGSK4 fusion protein. The expression of the His-OsGSW3.2 and GST-OsGSK4 fusion proteins and in vitro binding experiments were performed as described previously (Wang et al., 2009).

Haplotype analysis across the *OsGSW3.2* coding region and promoter region

Seven non-synonymous SNPs in the coding region of *OsGSW3.2* and eight non-synonymous SNPs in the promoter region of *OsGSW3.2* between KJ01 and HY3 were subjected to haplotype analysis via the rice 3K project (RFGB, https://www.rmbreeding.cn/Index/). Boxplots for grain length, width, length–width ratio, and weight were generated from data from *indica* and *japonica* rice cultivars of Asian origin. The haplotypes of more than four accessions were selected, and seven sites of variation were analyzed. The assay method was performed according to previous methods (Bai et al., 2023). The violin plot was generated with GraphPad.

Statistical analyses

All experiments were carried out with at least three independent biological replicates. Differences between samples were analyzed via Student's *t* test via the GraphPad Prism 5 software package. The trait analysis of the violin plot was performed via Tukey's honestly significant difference test in GraphPad Prism 5.

The primers used for these experiments are listed in Table S7.

AUTHOR CONTRIBUTIONS

XL participated in designing the research plans and performed the experiments and analysis; YL participated in

transcriptomic analysis, the haplotype analysis, and cytological analysis of the spikelets glumes; MT, WJ participated in the experiment of labeling the flowering seeds; YZ performed the KO vector construction; ZC read and commented on the manuscript; XL participated in the experimental design and conception; LW conceived the original idea, designed the research plan, supervised the research work, analyzed experimental data, wrote, and revised the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

RNA-Seq data in this study are openly available in the CNGB Nucleotide Sequence Archive (CNSA: https://db.cngb.org/cnsa) under the accession number CNP0006912. Other relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Brassinosteroid biosynthesis pathway.

Table S1. SNP variations of *OsGSW3.2* in the coding region of KJ01 and HY3 and their resulting amino acids.

Table S2. SNP variations of *OsGSW3.2* in the promoter region of KJ01 and HY3.

Table S3. Variation trait analysis of T_1 lines in the *OsGSW3.2* KO mutants and wild-type HY3.

Table S4. Grain size analysis of the *OsGSW3.2* OE mutants in the T_0 generation.

Table S7. All primer sequences used in the study.

Table S5. Differentially expressed genes between HY3 and KO for the 25% full-length spikelet hulls.

Table S6. Differentially expressed genes between HY3 and KO for the 50% full-length spikelet hulls.

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