Control of Grain Weight and Size in Rice (*Oryza sativa* L.) by *OsPUB3* Encoding a U-Box E3 Ubiquitin Ligase

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**Abstract**

Grain weight and size, mostly determined by grain length, width and thickness, are crucial traits affecting grain quality and yield in rice. A quantitative trait locus controlling grain length and width in rice, *qGS1-35.2*, was previously fine-mapped in a 57.7-kb region on the long arm of chromosome 1. In this study, *OsPUB3*, a gene encoding a U-box E3 ubiquitin ligase, was validated as the causal gene for *qGS1-35.2*. The effects were confirmed firstly by using CRISPR/Cas9-based mutagenesis and then through transgenic complementation of a Cas9-free knock-out (KO) mutant. Two homozygous KO lines were produced, each having a 1-bp insertion in *OsPUB3* which caused frameshift mutation and premature termination. Compared with the recipient and a transgenic-negative control, both mutants showed significant decreases in grain weight and size. In transgenic complementation populations derived from four independent *T*₀ plants, grain weight of transgenic-positive plants was significantly higher than transgenic-negative plants, coming with increased grain length and a less significant decrease in grain width. Based on data documented in RiceVarMap V2.0, eight haplotypes were classified according to six single-nucleotide polymorphisms (SNPs) found in the *OsPUB3* coding region of 4695 rice accessions. Significant differences on grain size traits were detected between the three major haplotypes, Hap1, Hap2 and Hap3 that jointly occupy 98.6% of the accessions. Hap3 having the largest grain weight and grain length but intermediate grain width exhibits a potential for simultaneously improving grain yield and quality. In another set of 257 *indica* rice cultivars tested in our study, Hap1 and Hap2 remained to be the two largest groups. Their differences on grain weight and size were significant in the background of non-functional *gse5*, but non-significant in the background of functional *GSE5*, indicating a genetic interaction between *OsPUB3* and *GSE5*. Cloning of *OsPUB3* provides a new gene resource for investigating the regulation of grain weight and size.

**Keywords:** Rice, Grain weight, Grain size, Milling quality, Ubiquitin-ligase enzyme, U-box domain

**Background**

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. Simultaneous improvement of rice grain yield and quality remains a difficult task for rice breeders as grain yield and quality are often negatively correlated with each other (Sakamoto et al. 2008). Grain weight and size, mostly determined by grain length, width and thickness, are crucial traits affecting grain quality and yield in rice (Zuo et al. 2014). Understanding the genetic and molecular basis for natural variation of grain weight and size is important for developing rice varieties with high yield and superior quality.

To date, causal genes for 25 quantitative trait loci (QTLs) regulating grain weight and size in rice have been cloned. They are located on all rice chromosomes except chromosomes 4, 11 and 12. The number of
genes located on chromosome 3 is the largest, with a total of eight genes. They are OsLG3 (Yu et al. 2017), OsLG3b/qLGY3 (Liu et al. 2018; Yu et al. 2018), GS3.1 (Zhang et al. 2021), GS3 (Fan et al. 2006), SG3 (Li et al. 2020), GL3.1 (Qi et al. 2012), GSA1 (Dong et al. 2020) and qTGW3 (Hu et al. 2018). Among them, OsLG3, OsLG3b/qLGY3 and GS3.1 are linked in the 4.3-Mb to 6.9-Mb region on the short arm, GS3 and SG3 are tightly linked in the 16.7-Mb to 16.9-Mb pericentromeric region, and the other three genes are located on the long arm. Four genes, GW6 (Shi et al. 2020), TGW6 (Ishimaru et al. 2013), GW6a (Song et al. 2015) and GL6 (Wang et al. 2019a), are located on chromosome 6, of which GW6 is located on the short arm and the other three are closely linked in the 26.0-Mb to 27.6-Mb region on the long arm. Followed are three genes on two chromosomes, including GW2 (Song et al. 2007), GS2/GL2/GLW2 (Hu et al. 2015; Che et al. 2016; Li et al. 2016) and TGW2 (Ruan et al. 2020) on chromosome 2, and GS5 (Li et al. 2011), GWS/GSE5 (Duan et al. 2017; Liu et al. 2017) and qGL5 (Qiao et al. 2021) on chromosome 5. Then are two genes on two chromosomes, including GLW7 (Si et al. 2016) and GL7/GW7 (Wang et al. 2015a, 2015b) on chromosome 7, and GW10 (Zhan et al. 2021) and GL10/OsMADS56 (Zhan et al. 2022; Zuo et al. 2022) on chromosome 10. The remaining three are qTGW1.2b (Chan et al. 2021), GW8 (Wang et al. 2012) and GS9 (Zhao et al. 2018) on chromosomes 1, 8 and 9, respectively. Of these, GS3 and GW5 were earlier isolated as major QTLs controlling grain length and grain width, respectively (Fan et al. 2006; Weng et al. 2008). More recently, GSE5 was confirmed as the causal gene for GW5 (Duan et al. 2017; Liu et al. 2017).

Rice grain quality parameters are usually classified into four categories, i.e. appearance quality, milling quality, cooking and eating quality, and nutritional quality. The appearance and milling quality are closely related to the grain size traits (Xie et al. 2013; Bao et al. 2019). Among the 25 cloned QTLs for grain size, pleiotropic effects with unfavorable association between grain yield and appearance and/or milling quality have been reported. The gw2w3 allele showed positive effects on grain yield by increasing grain width (GW) and thousand grain weight (TGW), but had negative effects on grain quality by increasing chalky rice percentage (CRP) and decreasing brown rice percentage and milled rice percentage (Song et al. 2007). The G52b, gw2p3–11 and GLW7 alleles are favorable for increasing grain length (GL), GW and TGW, but unfavorable for enlarging CRP (Hu et al. 2015; Si et al. 2016; Ruan et al. 2020). The GW8 allele is favorable for increasing GW and TGW but unfavorable for increasing endosperm transparency and increasing CRP (Wang et al. 2015a).

Ubiquitination is a type of post-translational modification of proteins, widely occurring in eukaryotic cells. It plays critical roles in controlling the synthesis and degradation of proteins in plant growth, development and responses to biotic and abiotic stresses. The ubiquitination system includes a cascade of enzymatic reactions determining by three main enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase enzyme (E3). The E3 ligase can be divided into two classes: the Homologous to E6-associated protein Carboxyl Terminus type and the Really Interesting New Gene (RING)/U-box type (Mao et al. 2022). GW2 encoding a RING-type protein with E3 ubiquitin ligase activity showed a major effect on grain size (Song et al. 2007). In addition, 77 annotated genes for U-box type E3 ligases of eight groups were found in rice genome (Zeng et al. 2008).

In our previous study, one QTL, qGS1-35.2 controlling GL and GW difference between indica rice varieties Zhenshan 97 (ZS97) and Milyang 46 (MY46), was fine-mapped in a 57.7-kb region one the long arm of chromosome 1 (Dong et al. 2018). In this study, the most likely gene for qGS1-35.2, Os01g0823900 encoding the U-box E3 ubiquitin ligase OsPUB3, was validated using CRISPR/Cas9-based gene knock-out (KO) and transgenic complementation of the KO mutant. Knock-out of OsPUB3 caused a decrease in grain weight and size but an increase in head rice. Correspondingly, transformation of a functional OsPUB3 allele into the KO mutant resulted in increased grain weight and size. Our finding provides a new gene resource to improve grain size and quality.

Results

Genetic Effects of OsPUB3 Identified Using CRISPR/Cas9-Based Mutagenesis

OsPUB3 KO mutants were produced for validating the effects of OsPUB3. Using the CRISPR/Cas9 system, two independent T0 homozygous mutants (KO-1 and KO-2) were produced from the recipient Zhonghui 161 (ZH161), an indica rice cultivar. Both the mutants had a 1-bp insertion at +91 in the coding region (Fig. 1A). The sequence variation introduced a frameshift mutation at the 31st amino acid (AA) and led to a premature translation termination at the 263rd AA. Finally, the number of amino acid residues changed from 680 to 262 for the two mutants (Fig. 1B).

T1 and T2 populations of the two mutants were tested against the recipient ZH161 and a transgenic negative control (CK). The T1 trial was conducted in 2019, in which 20 plants of each population were measured for
Fig. 1 Variations of the OsPUB3 knock-out mutants. A Variations of the DNA sequences in the target region. The protospacer adjacent motif site is shown in blue. Insertion is indicated by lowercase letter in red. B Variations of the predicted amino acid sequences. Letters in red indicate amino acid sequences of frameshift mutation. U-box domain is in green. Armadillo repeat domain is in blue. ZH161 is the recipient. CK is the transgenic-negative control. KO-1 and KO-2 are two homozygous knock-out mutants.
TGW, GL and GW. The T2 trial was conducted in 2021, using a randomized complete block design with three replications. A total of ten traits were measured, including TGW, GL, GW, number of panicles per plant (NP), number of grains per panicle (NGP), grain yield per plot (GY), brown rice recovery (BRR), milled rice recovery (MRR), head rice recovery (HRR), and heading date (HD). Phenotypic differences among the four genotypes were analyzed using the Duncan’s multiple range test. The results are presented in Table S1.

Compared with ZH161 and CK, the two mutants showed significant decreases in TGW and GW in both the T1 (Fig. 2A, C) and T2 (Fig. 2D, F) trials. Meanwhile, non-significant differences were observed between ZH161 and CK. For TGW, decreases over CK in T1 and T2 were 1.73 g (9.0%) and 2.09 g (10.1%) in KO-1, and 0.76 g (3.9%) and 1.13 g (5.4%) in KO-2, respectively (Additional file 2: Table S1). For GW, decreases over CK in T1 and T2 were 0.083 mm (3.4%) and 0.059 mm (2.3%) in KO-1, and 0.093 mm (3.8%) and 0.095 mm (3.7%) in KO-2, respectively. Decreases of TGW and GW due to OsPUB3 knock-out were evident, although the effect found in KO-1 was much stronger on TGW and slightly weaker on GW than that detected in KO-2.

Results on GL were much less consistent. Significant differences were observed between the two controls themselves, and changes over the controls varied greatly between KO-1 and KO-2. KO-1 remained to significantly decrease over ZH161 and CK in both the T1 (Fig. 2B) and T2 (Fig. 2E) trials. The decreases over CK were 0.265 mm (3.4%) in T1 and 0.433 mm (5.3%) in T2. In terms of the percentage change, KO-1 had a greater decrease in GL than in GW. On the other hand, KO-2 had a significant increase over CK in T1 (Fig. 2B) with a small value of 0.070 mm (0.9%), and a non-significant change in T2 (Fig. 2E). It could be concluded that knock-out of OsPUB3 caused a clear effect for decreasing grain weight, but the effect on the two component traits, grain length and width, was more variable.

To investigate whether cell proliferation or expansion is the source for the changes in grain weight and size, T2 samples of CK and KO-1 were measured and compared for the length, width and number of the outer glume epidermal cells. Non-significant difference was detected on the cell length and width, but the cell numbers in both the longitudinal and transverse directions were higher in CK than in KO-1 (Additional file 1: Figure S1). These results indicate that OsPUB3 affects grain length and width by controlling cell division.

Among the seven traits that were only tested in the T2 trial, significant differences among genotypes were detected on three traits, including NGP, BRR and HRR. For NGP, significant difference was only detected between KO-1 and CK (Fig. 2H), with grain number decreased by 22.9 in KO-1. For BRR, significant difference was only detected between KO-2 and CK (Fig. 2I), with the trait value decreased by 0.8% in KO-2. For HRR, the two mutants showed significant increases over ZH161 and CK. Compared with CK, the trait values in KO-1 and KO-2 increased by 13.0% and 11.4%, respectively. Non-significant difference was detected on the remaining four traits, including NP, GY, MRR and HD (Fig. 2G, I, K and M).

These results indicate that OsPUB3 is the causal gene for qGS1-35.2. This gene had significant genetic effects on grain size, and simultaneously influenced the critical trait of rice milling quality, HRR. Changes in grain size traits and HRR due to OsPUB3 knock-out are obvious (Additional file 1: Figure S2).

**Genetic Effects of OsPUB3 Identified Using Transgenic Complementation**

Genetic effects of OsPUB3 on grain size were further tested using transgenic complementation of a knock-out mutant. As described in the knock-out trial, the effect on grain size due to OsPUB3 knock-out was larger and more stable in KO-1 than in KO-2. Thus, KO-1 was targeted for transgenic complementation. A Cas9-free mutant, TC3-6, was selected from progenies of KO-1 and transformed with the OsPUB3 gene isolated from MY46, the male parent of the original mapping population. Four independent T0 transformants were identified and the resulting T1 populations were tested in 2021. Results on the phenotypic differences between transgenic-negative and transgenic-positive plants, tested by using the Student’s t-test, are presented in Table 1.

In each population, transgenic-positive plants were higher in TGW and GL compared with transgenic-negative plants. Changes in the trait values were also similar among the four population, ranging as 0.63–0.95 g (3.5–5.4%) for TGW, and 0.088–0.150 mm (1.2–2.0%) for GL. For GW, changes in populations CP-1, CP-3 and CP-4 were significant, with the positive plants decreased by
Fig. 2 (See legend on previous page.)
0.027–0.040 mm (1.1–1.6%) compared with the negative plants. In CP-2, the positive group also showed decreased GW compared with the negative group, but the difference was statistically non-significant.

Allelic Variation of OsPUB3 and Its Association with Grain Size

To analyze the allelic variation of OsPUB3 in rice germplasm accessions, we search the sequence variations in the coding region of OsPUB3 using the RiceVarMap V2.0 system (Zhao et al. 2015, 2021). Six single-nucleotide polymorphisms (SNPs) were found in the 4695 rice accessions including 2751 Indica, 1498 Japonica, 177 intermediate and 269 Aus. Eight haplotypes were classified and ordered following the number of accessions contained (Additional file 2: Table S2). The number of accessions included in Hap1, Hap2 and Hap3 accounted for 64.5%, 21.7% and 12.4% of the total, summing up to 98.6%. Based on our sequencing data of the three rice cultivars used in the map-based cloning of OsPUB3, ZS97 has Hap2, and MY46 and ZH161 have Hap1.

Then, we downloaded phenotypic data of TGW, GL, GW and ratio of GL to GW (RLW) documented in RiceVarMap V2.0. For haplotypes 4 to 8, the number of accessions having phenotypic data in each haplotype was less than 5, so we only compared phenotypic differences among Hap1, Hap2 and Hap3. As shown in Additional file 1: Figure S3, significant differences were detected on all the four traits. For TGW, the difference between Hap2 and Hap3 was non-significant, but they showed significant increase over Hap1. For GL, the three haplotypes were all significantly different, ranking from high to low as Hap3, Hap1 and Hap2. For GW, the three haplotypes were also all significantly different, ranking from high to low as Hap2, Hap3 and Hap1. For RLW, the difference between Hap1 and Hap3 was non-significant, but they showed significant increase over Hap2. Regarding MY46-type (Hap1) and ZS97-type (Hap 2) significant differences were observed on all the four traits. Compared with Hap2, Hap1 was lower in TGW and GW but higher in GL and RLW.

Since both ZS97 and MY46 are typical indica rice varieties, we further analyzed allelic variation of OsPUB3 in 257 indica rice cultivars selected from the National Mid-term Genebank for Rice at the China National Rice Research Institute (Additional file 2: Table S3). They contained 119 improved varieties and 53 landraces in China and 85 cultivars introduced from other countries. Four of the haplotypes mentioned above, from Hap1 to Hap4, were identified in these varieties (Additional file 2: Table S2). The largest and second largest groups remained to be the MY46-type (Hap1) and ZS97-type (Hap 2), containing 230 (89.5%) and 19 varieties (7.4%), respectively. The remaining two haplotypes had a total of 8 varieties.

The 257 varieties were grown in Lingshui (LS) and Hangzhou (HZ). Three traits, TGW, GL and GW, were measured. Phenotypic differences between the two haplotypes were tested using the Student’s t-test. In both the LS and HZ trails, TGW and GW were significantly higher in ZS97-type than in MY46-type, but the differences on

### Table 1  Genetic effects of OsPUB3 in four transgenic complementation populations

| Name | No. of plants | Trait | Mean ± SD | ± (P-N)b | ± (P-N)%c |
|------|--------------|-------|-----------|---------|-----------|
|      |    |       | Negative | Positive |         |         |
|      |    |       | Negative | Positive |         |         |
| CP-1 | 19 | TGW   | 18.16 ± 0.86 | 18.79 ± 0.99 | 0.63** | 3.5      |
|      |    | GL    | 7.659 ± 0.150 | 7.747 ± 0.196 | 0.088* | 1.2      |
|      |    | GW    | 2.494 ± 0.040 | 2.467 ± 0.035 | -0.027** | -1.1      |
| CP-2 | 28 | TGW   | 17.47 ± 0.68 | 18.41 ± 0.72 | 0.95**** | 5.4      |
|      |    | GL    | 7.461 ± 0.150 | 7.611 ± 0.151 | 0.150**** | 2.0      |
|      |    | GW    | 2.487 ± 0.035 | 2.472 ± 0.052 | -0.014 | -0.6      |
| CP-3 | 21 | TGW   | 18.62 ± 0.54 | 19.53 ± 0.83 | 0.91**** | 4.9      |
|      |    | GL    | 7.806 ± 0.144 | 7.912 ± 0.171 | 0.107** | 1.4      |
|      |    | GW    | 2.484 ± 0.029 | 2.444 ± 0.047 | -0.040*** | -1.6      |
| CP-4 | 25 | TGW   | 19.27 ± 0.85 | 19.97 ± 0.78 | 0.70*** | 3.6      |
|      |    | GL    | 7.911 ± 0.194 | 8.014 ± 0.183 | 0.103** | 1.3      |
|      |    | GW    | 2.510 ± 0.039 | 2.471 ± 0.053 | -0.039*** | -1.6      |

*a* TGW, 1000-grain weight (g); GL, grain length (mm); GW, grain width (mm)

*b* Increase or decrease of transgenic-positive plants over transgenic-negative plants

*c* Percentage increase or decrease of transgenic-positive plants over transgenic-negative plants

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001
GL were not significant (Additional file 1: Figure S4). This result indicates that the effect of OsPUB3 on TGW is mainly through increasing GW. Among the cloned grain size genes, GSE5 is a major gene controlling grain width, so we analyzed the effects of OsPUB3 in the background of functional GSE5 and non-functional gse5, respectively. As shown in Fig. 3, the effects of OsPUB3 on TGW and GW were significant in the gse5 background but non-significant in the GSE5 background. This result indicates that there is a genetic interaction between OsPUB3 and GSE5. The OsPUB3 showed larger effects on grain size in the non-functional gse5 background.

Discussion
In this study, we confirmed that OsPUB3 is the causal gene underlying a QTL for grain weight and size in rice. The effects were confirmed firstly by using CRISPR/Cas9-based mutagenesis and then through transgenic complementation of a Cas9-free KO mutant. One-bp insertion in OsPUB3 caused frameshift mutation and premature termination, bringing about decreases in grain weight and size. Transgenic complementation of OsPUB3 resulted in increased grain weight that came with increased grain length and a less significant decrease in grain width. Decreased grain weight and size owing to gene knock-out is accordance with increased grain weight and size due to genetic complementation, indicating that OsPUB3 is a gene positively regulating grain weight and size in rice.

In this study, OsPUB3 shows a clear influence on TGW in both the knock-out and complementation experiments. However, the control of OsPUB3 on GL and GW is not clear. For GL, KO-1 significantly decreased, but KO-2 showed a significant increase or non-significant change, and a significant difference between ZH161 and CK was also found. For GW, both KO-1 and KO-2 showed significant decreases, but the transgenic complementation plants showed a less significant decrease rather than increase. To investigate possible influences of the genetic background, these four materials were examined by using whole-genome re-sequencing. Variations were found in some annotated genes, but none of them was found either in the cloned genes for grain weight and size or in the off-target loci predicted by the CRISPR-GE (http://skl.scau.edu.cn). More work is required to determine whether the variation observed on the effect of OsPUB3 is related to interactions between OsPUB3 and other background genes.

Genetic effects of minor genes traits are easily disturbed by major genes. For example, one minor gene, SG3, had a substantial effect on grain length and weight
only when the major gene GS3 is not functional (Li et al. 2020). A similar result was found in our study on OsPUB3 and GSE5. The differences on grain weight and size between the two parental haplotypes of OsPUB3 were significantly in the background of non-functional gse5, but non-significant in the background of functional GSE5, indicating a genetic interaction between OsPUB3 and GSE5. We also analyzed the relationship between OsPUB3 and GS3. Interactions between the two genes were not evident, and their effects were additive with each other (data not shown).

OsPUB3 encodes a U-box/ARM repeat protein possessing E3 ubiquitin ligase activity. The U-box domain containing approximately 70 amino acid residues is a modified RING-finger domain, which could recruit E2 proteins for ubiquitination of pre-mRNA splicing complexes and unfolded proteins associated with the proline-isomerase chaperone, respectively (Aravind et al. 2000). The ARM repeat containing approximately 40 amino acid residues is a short leucine-rich protein-interacting domain, which serve to recognize and recruit the substrate (Zeng et al. 2008). So far, genetic effects of the other regions on U-box/ARM E3 ligase have not been reported. In this study, six SNPs were found in the coding region of OsPUB3. Four of them caused amino acid changes. Compared with Nipponbare (Hap2), SNP1 (C97T), SNP3 (C755T), SNP4 (A830G) and SNP5 (A1546T) led to amino acids change from Pro to Ser and Thr to Met (T516S), Thr to Gly (A1546T). The SNP4 is located in the U-box domain and the other three SNPs are not located in either the U-box domain or ARM. The SNP2 is not shown, and their effects were additive with each other (data not shown).

As shown in Fig. 2, decreases of GW and TGW in the two KO mutants led to decreases in grain yield per plot, although the change was statistically non-significant. On the other hand, the two KO mutants showed higher HRR that is an indication of better milling quality. These results are in agreement with the understanding that rice grain yield and quality traits are often negatively correlated with one another (Sakamoto et al. 2008). However, with the cloning of more and more genes regulating component traits of rice grain yield and quality, it has been shown that the negative correlation between yield and quality can be overcome by combining complementary genes. For instance, combining the GW7^{TPA} allele with gw8^{bas-mat} allele (Wang et al. 2015a), or OsMADS7^{lev3} allele with dep1-1 and gs3 alleles could simultaneously improve rice grain yield and quality (Liu et al. 2018). In addition, creation of new allelic variations through genome editing also provided a novel strategy to solve negative correlations between different traits. For IPA1 as a rice gene increasing grains per panicle but decreasing tillers (Jiao et al. 2010), a novel allele ipa1-pro10 simultaneously increasing grains per panicle and tillers was created using a tiling-deletion-based CRISPR-Cas9 screen (Song et al. 2022). Among the three major haplotypes of OsPUB3, Hap3 has the intermediate grain width but largest grain weight and grain length (Additional file 1: Figure S3). Thus, a favorable slender grain shape may be accompanied by a higher grain weight. Therefore, this haplotype exhibited a potential in rice breeding for simultaneously improving rice grain yield and quality.

It is often observed that genes controlling the same trait are closely linked. Cloned genes showing major effects on rice grain weight and size have provided clear examples. Two gene clusters for GL on chromosome 3, OsLG3 and OsLG3b/qLGY3, and GS3 and SG3, are located in a 1.6-Mb region on the short arm and a 0.2-Mb region in the pericentromeric region, respectively (Fan et al. 2006; Yu et al. 2017, 2018; Liu et al. 2018; Li et al. 2020). TGW6, GW6a and GL6 for grain weight are in a 1.6-Mb region on the long arm of chromosome 6 (Ishimaru et al. 2013; Song et al. 2015; Wang et al. 2019a). In our previous studies, four minor-effect QTLs for grain size and its component traits, including qTGW1.2a, qTGW1.2b, qGS1-35.2 and qGW1-35.5, were dissected in a 4.5-Mb region on the long arm of chromosome 1 (Wang et al. 2015c; Dong et al. 2018; Wang et al. 2019b). We reported the cloning of qTGW1.2b in 2021 (Chan et al. 2021) and that of qGS1-35.2 here. Recently, Shang et al. (2022) reported that LOC_Os01g57250 encoding an unknown expressed protein is the likely causal gene underlying qTGW1.2a. These results indicate that minor-effect genes controlling the same trait are also generally close linked.

**Materials and Methods**

**Plant Materials**

Two sets of rice materials were used in this study. One contained OsPUB3 KO mutants, the other contained OsPUB3 transgenic complementation plants.
OsPUB3 KO mutants in the background of ZH161 were produced by using the CRISPR/Cas9 system. The recipient ZH161 has the same genomic sequence at OsPUB3 locus as MY46 which was the male parent of the mapping population for OsPUB3. Two independent T₀ homozygous transgenic plants, KO-1 and KO-2, were obtained. Both the mutants had a 1-bp insertion at +91 in the coding region (Fig. 1A). The T₁ and T₂ knock-out populations derived from the two homozygous mutants were used to test the genetic effects of OsPUB3 on grain size and other yield and quality traits.

OsPUB3 transgenic complementation plants were produced in the background of a Cas9-free progeny of the mutant KO-1. Four independent T₀ transgenic-positive plants were obtained. Each T₀ plant was selfed to produce a T₁ population segregating at the target gene locus. In each T₁ population, individual plants were genotyped and measured for grain size traits. The four T₁ complementation populations were used to verify that OsPUB3 is the cause gene for qGS1-35.2.

Field Experiment and Phenotyping
All the rice materials were grown in the paddy field of China National Rice Research Institute located at Hangzhou, Zhejiang Province, China. The plants were grown at a spacing of 16.7 cm between plants and 26.7 cm between rows. Field management followed local agricultural practice.

For the KO experiment, T₁ and T₂ plants of the two KO mutants were tested with the recipient ZH161 and a transgenic-negative control. The T₁ lines were grown in 2019, in which 20 plants were included for each of the four genotypes. At maturity, each plant was harvested and sun-dried. Three traits were measured, including TGW, GL and GW. Two samples of 100 fully filled grains were randomly selected for measurement using an electronic image analysis system (Model SC-G, Wanshen Ltd., Hangzhou, Zhejiang Province, China). The T₁ lines were grown in 2021, in which each population were used to verify the sequence variation on grain size and other yield and quality traits.

For the complementation experiment, four T₁ populations were grown in 2021, in which each population included 108 plants. At maturity, the three grain size traits, TGW, GL and GW, were measured based on a single-plant basis.

Construction of the Knock-Out and Complementary Vectors
For the KO experiment, the target site which was designed applying the web-based tool CRISPR-GE was located at +74 to +93 in exon 1 (Fig. 1A). Oligonucleotide Cri-PUB3 (Additional file 2: Table S4) was ligated into the CRISPR/Cas9 expression vector BGK03 following the manufacturer’s instructions (Biogle Co., Ltd., Hangzhou, China). The BGK03 vector comprised a rice U6 promoter for activating the target site sequence, a Cas9 gene driven by the maize ubiquitin promoter and a hygromycin marker gene driven by Cauliflower mosaic virus 35S promoter. The expression vector was introduced into ZH161 using Agrobacterium tumefaciens-mediated transformation.

For the complementation experiment, a DNA fragment of 6176 bp containing the 2879-bp promoter, the 2043-bp coding region, and the 1254-bp termination site was amplified from the genomic DNA of MY46 and cloned into binary vector pCAMBIA1300. The pCAMBIA1300 vector comprised a kanamycin resistance gene for bacterial selection and a hygromycin resistance gene for plant selection. The hygromycin resistance gene was driven by a double-enhancer version of the Cauliflower mosaic virus 35S promoter. The target gene expression was driven by its own promoter. The expression vector was introduced into Tc3-6, a Cas9-free progeny of KO-1 using Agrobacterium tumefaciens-mediated transformation.

Detection of Transgenic Plants
Genomic DNA of the T₀ plants was extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For the KO experiment, T₀ transgenic plants were identified by using the hygromycin gene marker Hyg. The OsPUB3 gene fragment surrounding the target region was amplified from the Hyg-positive plant using the primer pairs Seq-PUB3. The PCR fragments were directly sequenced by Sanger method and decoded using the web-based tool DSDecodeM (http://skl.scau.edu.cn/dsdecode). Two independent T₀ homozygous mutants were obtained. Each T₀ mutant was selfed to produce a T₁ population. Eight plants from each T₁ population were used to verify the sequence variation by sequencing. For the complementation experiment, T₀ transgenic plants were also identified by using the hygromycin gene marker Hyg. Four independent Hyg-positive plants were obtained. Each of them was selfed to produce a T₁ population. In each resultant T₁ population, DNA of each plant was extracted from young leaves using a mini-preparation method (Zheng et al. 1995). Individual plants
of each T1 population were genotyped using the primer pairs Seq-PUB3 that was used to detect the sequence variation of the target region for knockout mutants. Primers are listed in Additional file 2: Table S4.

Data Analysis
For the KO experiment, the Duncan’s multiple range test was employed to test the phenotypic differences among the KO mutants, transgenic-negative control and recipient. Any two groups having a common letter, are not significantly different at a 0.05 level of significance. Instead, any two groups with different letters are significantly different at a 0.05 level of significance. For the complementation experiment, the Student’s t-test was employed to test the phenotypic differences (p < 0.05) between transgenic negative and positive segregants.

Microscopy Observation
CK and KO-1 were used for observation of outer glume epidermal cell. Young spikelet hulls were fixed with 2.5% glutaraldehyde for 24 h and then dehydrated in a graded series of ethanol (30%, 50%, 75%, 95%, 100%, 100% and 100%). The dehydrated sample were coated with gold–palladium using ion sputter (Model E-1010, Hitachi, Japan) and observed using scanning electron microscope (Model TM-1000, Hitachi, Japan). Cell length and width of the outer glumes were measured, then cell number in the transverse and longitudinal direction were calculated. Twenty glumes were used for CK and KO-1 respectively. The Student’s t-test was employed to test the differences in cell length, width and numbers between CK and KO-1 (p < 0.05).

Haplotype Analysis of OsPUB3
Haplotype analysis was firstly analyzed by using data documented in RiceVarMap V2.0 database (http://ricevar.armap.ncpgr.cn). Data on the genomic variations in the OsPUB3 coding region of all rice accessions in the database was obtained by using the “Search for Variation by Gene” function. Data on the OsPUB3 haplotypes were downloaded from the website.

The allelic variation of OsPUB3 was further analyzed using 257 indica rice cultivars. Total DNA was extracted from a single plant of each germplasm using the method of Zheng et al. (1995). The fragment covering the OsPUB3 gene was amplified using the primer pairs S3900. The PCR fragments were directly sequenced by Sanger method. Sequences were aligned by Clustal W program (University College Dublin, Dublin, Ireland) and analyzed with MEGA 6.0 software (Mega Ltd., Auckland, New Zealand). For GSE5, one primer pairs, GSE5-Del, was designed according to the sequence variation in the promoter of GSE5 (Duan et al. 2017).

Conclusion
Decreased grain weight and size owing to gene knock-out is accordance with increased grain weight and size due to genetic complementation, indicating that OsPUB3 is a gene positively regulating grain weight and size in rice. Genetic interaction between OsPUB3 and GSE5 was detected. The OsPUB3 showed larger effects on grain weight and size in the non-functional gse5 background but not in the functional GSE5 background. Cloning of OsPUB3 provides a new gene resource for investigating the regulation of grain size and its utilization in rice breeding.

Abbreviations
BRR: Brown rice recovery; HD: Heading date; HRR: Head rice recovery; GL: Grain length; GW: Grain width; GY: Grain yield per plot; MIR: Milled rice recovery; MY46: Milyang 46; NGP: Number of grains per panicle; NP: Number of panicles per plant; QTL: Quantitative trait locus; TGW: 1000-Grain weight; ZH161: Zhonghu 161; Z597: Zhenhan 97.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12284-022-00604-1.

Additional file 1 Figure S1. Characterization of the cells in the outer glume epidermal of CK and KO-1 for OsPUB3. A Comparison of cell length in the outer glume epidermal. B Comparison of cell width in the outer glume epidermal. C Comparison of cell numbers in the longitudinal direction. D Comparison of cell numbers in the transverse direction. E Scanning electron microscopic images of the cells. Scale bars=100 μm. Values are given as the mean ± SD (n = 20). Differences between CK and KO-1 were tested by student’s t-test. *, p < 0.05; ns: not significant. Figure S2. The effects of OsPUB3 knock-out on grain weight, grain length and head rice recovery. A Comparisons of grain width among the knock-out mutants and the two controls. B Comparisons of grain length among the knock-out mutants and the two controls. C Comparisons of head rice recovery among the knock-out mutants and the two controls. Scale bars = 10 mm. Figure S3. Phenotypic differences among three haplotypes of OsPUB3. A 1,000-grain weight. B Grain length. C Grain width. D Ratio of grain length to width. Values are given as the mean ± SD (n = 338 for Hap1, n = 122 for Hap2, n = 50 for Hap3). Values with different letters are significantly different at p < 0.05 based on the Duncan’s multiple range test. Figure S4. Phenotypic differences between Z597-type and MY46-type of OsPUB3. A-C Three traits measured in Hangzhou trial. D-F Three traits measured in Hangzhou trial. Values are given as the mean ± SD (n = 19 for Z597-type; n = 230 for MY46-type). *, p < 0.05; **, p < 0.01; ns: not significant. Figure S5. Protein variations of the eight haplotypes. Difference in amino acid is shown in green.

Additional file 2 Table S1. Phenotypic change due to OsPUB3 knockout. Table S2. Haplotypes of OsPUB3 in rice germplasms. Table S3. Rice germplasms used in this study. Table S4. Primers used in this study.

Acknowledgements
We thank the RiceVarMap v2.0 for providing the genotype and phenotype of the rice germplasm accessions used in this study.

Author contributions
ZJY, ZYI and YLY designed the experiments; WSL, ZZH and ZYJ conducted the populations; WSL, ZZH and FYY performed genotyping; WSL, HDR and ZYJ conducted phenotyping. ZYI and ZJY analyzed the data. ZJY and ZYI wrote the manuscript. All authors read and approved the final manuscript.
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