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Letter to the Editor

TGW3, a Major QTL that Negatively Modulates Grain Length and Weight in Rice

Dear Editor,

Grain length (size) and weight are essential components of crop yield. To date, many QTLs/genes for these traits have been identified. *GS3* encodes a putative transmembrane protein and functions as a negative regulator, and its larger-grain allele contains a nonsense mutation causing a 178-aa truncation (Fan et al., 2006). *GL3.1/qGL3* encodes a putative protein phosphatase and also acts as a negative regulator of grain size (Qi et al., 2012; Zhang et al., 2012). Another negative regulator of grain size and weight is TGW6, which hydrolyzes indole-3-acetic acid (IAA)-glucose into IAA and glucose (Ishimaru et al., 2013). In contrast, *GW6a* is a positive regulator of grain weight, which encodes a novel histone H4 acetyltransferase (Song et al., 2015). Copy number variation at the *GL7/GW7* locus causes elevated expression of *GL7* and thus an increase in grain length (Wang et al., 2015a, 2015b). *GL2/GS2* encodes the plant-specific transcription factor OsGRF4, and its larger-grain allele harbors a mutation preventing cleavage by miR396c, resulting in elevated *GL2/GS2* expression (Hu et al., 2015; Che et al., 2016). *GLW7* encodes the plant-specific transcription factor OsSPL13, and high *OsSPL13* expression is associated with larger grains (Si et al., 2016). These findings have greatly enhanced our understanding of grain length and weight regulation; however, there are still gaps in integrating these factors into genetic network(s). Here, we report a thorough dissection of the QTL composition of grain size and the characterization of a novel QTL, *qTGW3*, that regulates grain length and weight in rice.

In a previous study, to understand how super-large rice grains in the JZ1560 cultivar form, we performed QTL analyses and identified a single grain-length QTL on chromosome 6 (Ying et al., 2012). We assumed that there were also undetected major QTL(s) shaping grain size. We then produced an F7 RIL population derived from JZ1560 and the small-grain cultivar Huanghuazhan (HHZ) (Figure 1A). As expected, phenotypic variation in almost all investigated traits approximated a normal distribution, and we observed transgressive segregation for grain length (GL) and grain yield per plant (GY) (Supplemental Figure 1). We thus considered this population to be suitable for QTL mapping and inferred that novel QTL(s) or a combination of different loci contributed to the transgressive GL and GY phenotypes.

We utilized SLAF-seq technology for genotyping the RIL population, and ultimately obtained 18 194 efficient SLAFs to construct a genetic map (Supplemental Figure 2). Using this map and the GL, grain width (GW), thousand grain weight (TGW), and GY phenotypes measured in either 2015 or 2016, we mapped more than 40 QTLs, including the known loci *GW2* and *qSW5/GW5* (Ying et al., 2012). Most of the mapped GL, GW, and TGW QTLs (91%, i.e., 29 of 32) were detected repeatedly; however, only approximately 42% of the GY QTLs were detected more than once (Supplemental Figure 3 and Supplemental Table 1). To confirm these results, we performed PCR genotyping and QTL analysis and observed that most of the mapped QTLs, especially those with relatively larger effects, could be reproduced (Supplemental Figure 4). Thus, we have obtained a deeper understanding of the genetic architecture of the super-large grain accession JZ1560 and identified novel major QTLs, such as *qGL1-1*, *qGL3/qTGW3-2* (here named *qTGW3*) (see below) for grain size, weight, and yield.

We next scrutinized the *qTGW3* locus (Supplemental Figure 5A and 5B). We screened residual heterozygous lines (RHLs) that are heterozygous for *qTGW3* GL segregation in the RHL populations was consistent with single-gene regulation (Supplemental Figure 5C). We then bred a nearly isogenic line, NIL(*TGW3*), containing an introgression segment from JZ1560 between markers JD3014 and JD3015. Compared with the HHZ isogenic control, we observed a considerable increase in GL (7.6%) but a relatively smaller increase in GT (4.2%) and GW (1.1%) in NIL(*TGW3*) (Supplemental Figure 6A–6C); We also observed a significant increase (8.5%) in TGW (Supplemental Figure 6D). We thus concluded that the enhanced TGW in NIL(*TGW3*) was primarily due to an increase in GL.

We further compared the grain yields of NIL(*TGW3*) with the control plants and observed over 10% higher GY in NIL(*TGW3*) (Supplemental Figure 6E). We also examined whether *TGW3* has pleiotropic effects. The control and NIL(*TGW3*) plants did not differ in appearance and had indistinguishable panicle numbers (Supplemental Figure 6F), but NIL(*TGW3*) plants were slightly taller ($p < 0.05$). In addition, both total grain number per plant and empty grain number per plant were lower in NIL(*TGW3*) than in the control, but filled grain number per plant did not differ. More interestingly, the seed setting rate of NIL(*TGW3*) was significantly higher than that of the control ($p < 0.01$) (Supplemental Figure 7). These results suggest that *TGW3* affects other traits beyond its profound impact on grain size.

Detailed analysis of RHLs for *qTGW3* enabled us to map the target gene to an interval between JD3014 and JD3015, where a total of 54 recombinants were obtained (Figure 1B and 1C). Finally, we localized *qTGW3* to a region of 18.7 kb (Figure 1D), in which we identified three predicted ORFs as viable candidates for *qTGW3*. One of these ORFs, *LOC_Os03g62500* encodes a GSK3/SHAGGY-like kinase. A previous study revealed that the rice GSK3/SHAGGY-like kinase GSK2 plays an important role in grain size regulation (Tong et al., 2012). Sequence analysis showed that, compared with the HHZ (small-grain) allele of *LOC_Os03g62500*, the JZ1560 allele was

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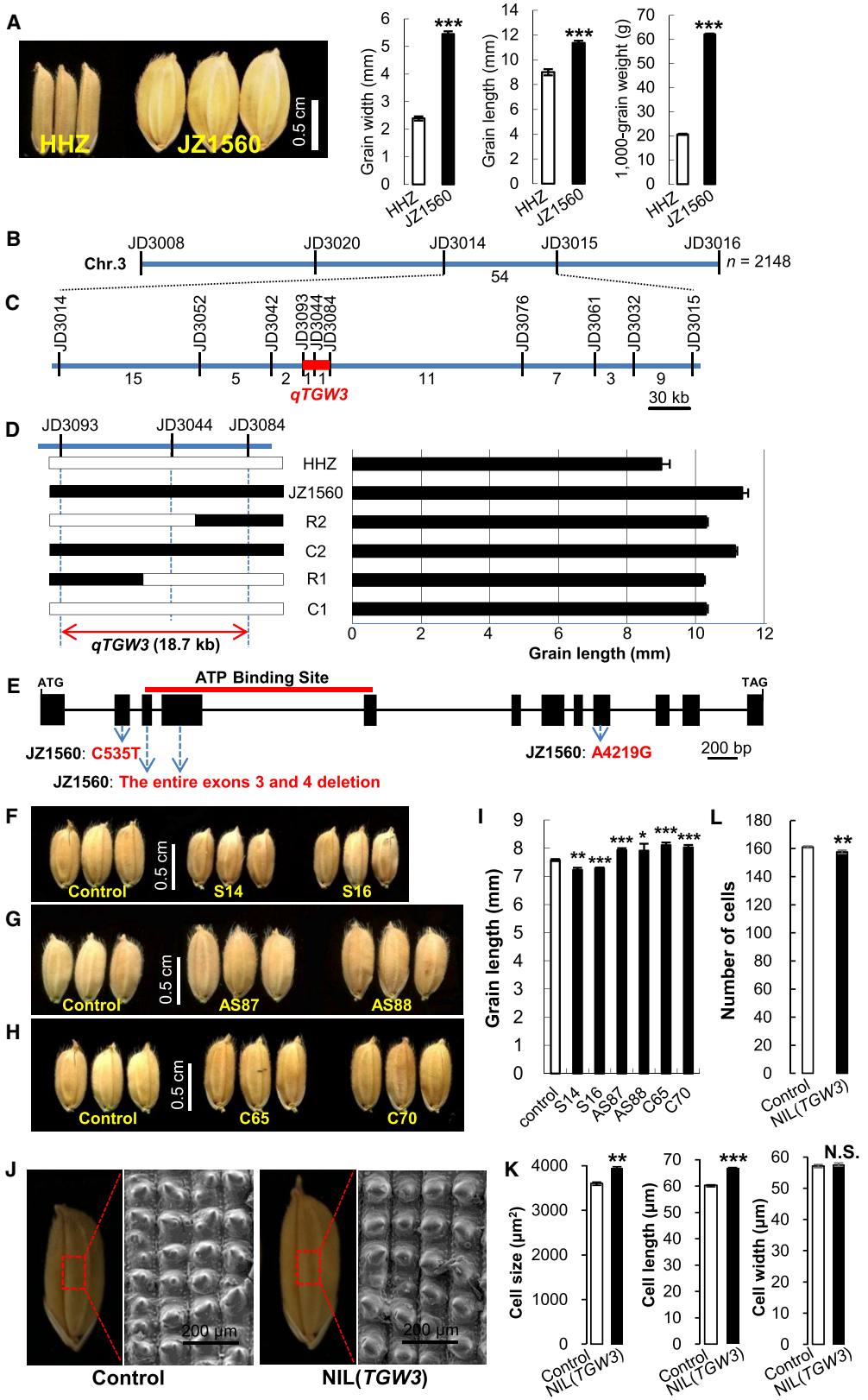

Figure 1. Cloning of QTL *qTGW3* and Its Control of Grain Size through Coordinated Alteration of Cell Size and Cell Number.

(A) Grain phenotypes of the small-seed accession HHZ and the super-large seed accession JZ1560.

(B) The *qTGW3* locus was initially mapped to chromosome 3 between molecular markers JD3014 and JD3015.

(legend continued on next page)

missing both exons 3 and 4, which encode an ATP binding site (ABS) domain (Figure 1E), suggesting that the large-grain allele could be, at least partially, functionally incapacitated. To test this hypothesis, we produced transgenic rice plants with altered *LOC_Os03g62500* gene expression; enhanced expression of the *LOC_Os03g62500* HHZ allele driven by the 35S promoter reduced GL (Figure 1F, Supplemental Figure 8A and 8B), while downregulation of endogenous gene expression resulted in enlarged grains (Figure 1G, Supplemental Figure 8C and 8D). In addition, mutation of the *LOC_Os03g62500* gene by CRISPR/Cas9 also caused enlarged grains (Figure 1H, Supplemental Figure 8E). Based on these results, we conclude that *LOC_Os03g62500* is the causal gene underlying the *TGW3*.

To reveal the molecular basis for *TGW3* regulation of grain size we examined its temporal and spatial expression profile using real-time PCR. *TGW3* was preferentially expressed in young panicles, which is consistent with its biological function (Supplemental Figure 9A). We also analyzed the subcellular localization of green fluorescent protein (GFP)-tagged TGW3 in transiently transformed tobacco (*Nicotiana benthamiana*) leaf epidermal cells, and found that the fusion protein localized to the nucleus and the cytoplasm (Supplemental Figure 9B).

We found two nucleotide substitutions in the cDNA sequence of the JZ1560 allele ($TGW3^{JZ}$) that did not cause any amino acid changes and a 333-bp deletion (equivalent to 111 amino acid residues) that corresponds to both exons 3 and 4 in the *TGW3* HHZ allele ($TGW3^{HHZ}$) (Figure 1E, Supplemental Figures 10 and 11A). Subsequent SWISS-MODEL database analysis indicated that the deletion resulted in the complete loss of the ABS domain and severe damage to the dimer interface domain (Supplemental Figure 11B), suggesting that the $TGW3^{HHZ}$ protein could form a dimerization structure, but $TGW3^{JZ}$ could not (Supplemental Figure 11C). We tested this hypothesis using yeast two-hybrid (Y2H) experiments and found that $TGW3^{HHZ}$ could interact with itself in yeast cells, but $TGW3^{JZ}$ could not (Supplemental Figure 11D). We also tested our prediction that altered nucleotides in the 3rd intron of the $TGW3^{JZ}$ allele was responsible for its distinct RNA splicing pattern. Indeed, *Agrobacterium*-mediated transient assays in *N. benthamiana* revealed that a fragment containing the first four exons and three introns of the $TGW3^{JZ}$ allele caused retention of the 3rd intron (Supplemental Figure 12); we inferred that intron retention would result in the complete loss of exons 3 and 4.

We next sought to uncover the cytological basis underlying the regulation of grain size by *TGW3*, and compared the center part of the spikelet hull (lemma) at maturity in NIL(*TGW3*) and the corresponding control using scanning electron microscopy (Figure 1J). The spikelet hull cells of NIL(*TGW3*) were larger in size (by 10.1%) and significantly longer (10.6%, in the grain-length orientation) than those of the control, although the cell width did not differ; however, the estimated total cell number was significantly lower in NIL(*TGW3*) (Figure 1K and 1L). We also inspected the cytological features in grains of transgenic rice with altered *TGW3* expression. Consistent with the above results, the sizes and lengths of cells in spikelet hulls of lines with downregulated and/or CRISPR-Cas9-edited *TGW3* expression were significantly higher, while those of lines overexpressing *TGW3* were clearly lower compared with the control (Supplemental Figure 13A and 13B). Furthermore, the estimated total cell number of grains from lines with reduced expression or with edited *TGW3* was significantly lower than that of the control, yet the cell number of plants overexpressing *TGW3* did not differ (Supplemental Figure 13C). Collectively, these results suggest that *TGW3* has contrasting effects on cell size and cell number, suggesting that it regulates grain size through coordinated alteration of cell expansion and cell division.

To investigate whether *TGW3* is a domestication-associated gene, we analyzed the genomic sequences of 1083 *O. sativa* and 446 *O. rufipogon* accessions. However, we did not detect any domestication signals in the 100 kb region surrounding the *TGW3* locus (Supplemental Figure 14A). We also analyzed the coding sequences of *TGW3* from Sancunli (SCL) and Changxiangdao (CXD), two other rice varieties that have longer grains, and found these sequences were almost identical to the JZ1560 allele (Supplemental Figure 14B). These observations suggest that *TGW3* might not be a domestication gene and that its large-grain allele is rare.

In summary, in this study, through in-depth dissection of the QTLs for grain size we identified more than 40 QTLs for grain size and yield. Importantly, most of them have not yet been cloned. We cloned a major QTL, *qTGW3*, and found that it

(C) High-resolution genetic mapping was conducted by analyzing a chromosome segment substitution line population of 2148 individuals segregating for the *qTGW3* target region.
(D) Progeny testing of fixed recombinant plants (F4) narrowed *qTGW3* to an 18.7-kb interval between molecular markers JD3084 and JD3093. Grain lengths (mean ± SD) of the recombinant line (R1) and control 1 (C1; homozygous for HHZ in the target region) did not differ, but the grain lengths of another recombinant line (R2) were significantly higher than those of control 2 (C2; homozygous for JZ1560 in the target region). Filled and open bars represent chromosomal segments homozygous for JZ1560 and HHZ, respectively.
(E) *TGW3* gene structure and mutation sites, including nucleotide substitutions and deletions in JZ1560. The location of the predicted ATP binding site domain is indicated by a red line. Black boxes represent exons; thin lines between exons represent introns. Boxes and lines are drawn to scale as indicated.
(F–I) Grain phenotypes of transgenic plants with overexpression of the HHZ allele **(F),** downregulated endogenous expression **(G)**, and a CRISPR/Cas9-edited form **(H)** of the LOC_Os03g62500 gene. Quantification of the grain-length traits in (**F** and **G**) and (**H**) is shown (**I**). S14 and S16 are transgenic lines carrying the sense HHZ *TGW3* cDNA; AS87 and AS88 are transgenics carrying antisense HHZ *TGW3* cDNA; and C65 and C70 are independent *TGW3* mutant lines generated by the CRISPR/Cas9 technology.
(J–L) The spikelet hull of NIL(*TGW3*) is longer and contains much larger but fewer cells than that of the HHZ isogenic control. **(J)** Images of spikelet hulls and cells obtained by scanning electron microscopy of NIL(*TGW3*) and the corresponding control. **(K)** Comparisons of cell size, length, and width, and **(L)** total cell number of the spikelet hulls in NIL(*TGW3*) and the HHZ isogenic control.
Data in **(A and I)** (n = 20) and **(K** and **L)** (n = 30) are means ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; N.S., not significant. Student's t-test was used to generate the p values.

encodes a negative regulator of grain length and weight and coordinately alters cell size and cell number in the spikelet hull. Our findings thus provide new insights into the genetic architecture of a super-large rice grain accession and uncover a novel mechanism for grain size regulation.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

J.-Z.Y., M.M., and C.B. conducted most of the experiments; X.-H.H. performed population sequence comparisons; J.-L.L. and Y.-Y.F. performed some of the experiments and experimental field management; X.-J.S. designed the experiments and wrote the paper.

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Jie-Zheng Ying[1,5], Ming Ma[2,4,5], Chen Bai[2,4,5], Xue-Hui Huang[3], Jian-Li Liu[2], Ye-Yang Fan[1] and Xian-Jun Song[2,]*

[1]National Key Laboratory of Rice Biology and Chinese Center of Rice Improvement, China National Rice Research Institute, Hangzhou 310006, China
[2]Key Laboratory of Plant Molecular Physiology, Institute of Botany, the Chinese Academy of Sciences, No.20 Nanxincun, Xiangshan, Beijing 100093, China
[3]College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, China
[4]Graduate University of Chinese Academy of Sciences, Beijing 100049, China
[5]These authors contributed equally to this article
***Correspondence: Xian-Jun Song (songxj@ibcas.ac.cn)**

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