The Rice HGW Gene Encodes a Ubiquitin-Associated (UBA) Domain Protein That Regulates Heading Date and Grain Weight

Juan Li1, Huangwei Chu2, Yonghong Zhang1, Tongmin Mou1, Changyin Wu1, Qifa Zhang1, Jian Xu1,2*
1 National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China, 2 Department of Biological Sciences and NUS Centre for BioImaging Sciences, National University of Singapore, Singapore, Singapore

Abstract

Heading date and grain weight are two determining agronomic traits of crop yield. To date, molecular factors controlling both heading date and grain weight have not been identified. Here we report the isolation of a hemizygous mutation, heading and grain weight (hgw), which delays heading and reduces grain weight in rice. Analysis of hgw mutant phenotypes indicate that the hemizygous hgw mutation decreases latitudinal cell number in the lemma and pala, both composing the spikelet hull that is known to determine the size and shape of brown grain. Molecular cloning and characterization of the HGW gene showed that it encodes a novel plant-specific ubiquitin-associated (UBA) domain protein localized in the cytoplasm and nucleus, and functions as a key upstream regulator to promote expressions of heading date- and grain weight-related genes. Moreover, co-expression analysis in rice and Arabidopsis indicated that HGW and its Arabidopsis homolog are co-expressed with genes encoding various components of ubiquitination machinery, implying a fundamental role for the ubiquitination pathway in heading date and grain weight control.

Introduction

Rice (Oryza sativa L.) is one of the most important food crops worldwide and a model for genetic and genomic researches in cereals [1–3]. With an ever-increasing global population but rapidly decreasing farmland and changing environment, there is an urgent need to maintain the yield stability of rice and further improve the rice grain yield using molecular genetic breeding approaches.

Heading date, often called flowering time, is an important agronomic trait that is particularly crucial for rice to adapt to different cultivation areas and cropping seasons, thus ensuring yield stability. Several genes involved in the photoperiod control of flowering time in rice have been recently identified (Figure S1) [4] and some of these showed sequence similarity to Arabidopsis flowering time genes. Heading date1 (Hd1), an Arabidopsis CONSTANS (CO) ortholog in rice, promotes flowering under short day (SD) conditions and represses it under long day (LD) conditions [5]. Heading date 3a (Hd3a), a rice ortholog of the Arabidopsis FLOWERING LOCUS T (FT) gene, is positively regulated by Hd1 [6–8]. Hd3a and its closest homolog Rice FT-like1 (RFT1) act redundantly to promote flowering [7–10]. Regulation of the Hd1/Hd3a module is mediated by OsGAI, a rice ortholog of GIGANTEA (GI) [11], which acts in the LD flowering pathway upstream of CO and FT [12]. On the other hand, Early heading date1 (Ehd1), a flowering time gene unique to rice, encodes a B-type response regulator promoting floral transition preferentially under SD conditions, even in the absence of functional alleles of Hd1 [13]. Expression analysis revealed that Ehd1 functions upstream of Hd3a and RFT1 [13], whereas under LD conditions, transcription of Ehd1 and Hd3a but not Hd1 is repressed by Ghd7 [14], which encodes a CCT domain protein and has major effects on an array of traits in rice, including number of grains per panicle, plant height and heading date. Enhanced expression of Ghd7 under LD conditions delays heading and increases plant height and panicle size [14]. In addition, Rice Indeterminate 1/Early heading date 2 (RID1/Ehd2) [15,16], encoding a Cys-2/His-2-type zinc finger transcription factor orthologous to the maize INDETERMINATE1 (ID1) gene [17], has been shown to regulate the floral transition in rice, similar to the ID1 function in maize. Mutations in RID1 or Ehd2 led to either never-flowering or extremely late flowering phenotype in rice [15,16]. Genes known to be involved in flowering time regulation, especially RFT homologs and those in the Ehd1/Hd3a pathway, are reduced to an undetectable level in these mutants [15,16], suggesting that RID1/Ehd2 acts as the master switch for the transition from the vegetative to reproductive phase, and promotes flowering upstream the photoperiod pathway in rice.

Rice yield potential was determined by three yield components, namely panicles per plant, grain weight and grain number. Grain weight, which is generally indicated as one-thousand-grain weight, is determined by the volume (size) and the plumpness (filling) of the grain [3]. Analyses and molecular cloning of quantitative trait loci
(QTLs) and rice mutants for grain weight have led to the identification of four genes, including Grain Size 3 (GS3), Grain Weight 2 (GW2), QTL for seed width on chromosome 5 (GW5/qSW5) and GRAIN INCOMPLETE FILLING 1 (GIF1) (Figure S2) [18–23]. GS3, a major QTL for grain length and weight and a minor QTL for grain width and thickness in rice, encodes a protein composed of four putative functional domains which differentially regulate grain size [18], likely through modulating cell division at the longitudinal direction [19]. GW2 encodes a cytosolic RING-type E3 ubiquitin ligase believed to negatively regulates cell division by targeting its substrates to the 26S proteasome for regulated proteolysis [20]. Loss of or reduced GW2 function increases cell numbers in the outer parenchyma cell layer of lemma and palea composing the spikelet hull and results in a wider spikelet hull, which in turn accelerates the milk filling rate of the grain and enhance the grain width, weight and yield [20]. GW5/qSW5, a QTL for grain weight and width consistently detected on chromosome 5, encodes a previously unknown nuclear protein, which physically interacts with a polyubiquitin [21,22]. Grains of the near-isogenic line (NIL) homozygous for the mutant allele resulted from a 1.2-kb deletion in the GW5 genomic are significantly heavier than the NIL homozygous for the wild type (WT) allele, primarily due to an increase in cell number in the outer glume [21,22]. GIF1 encodes a cell-wall invertase required for carbon partitioning during early grain-filling [23]. During grain-filling GIF1 has a more restricted expression pattern in cultivated rice than in the wild rice species, which produce smaller grains [23]. Constitutive or ectopic expression of the cultivated GIF1 using the 35S or rice Waxy promoter results in smaller grains, whereas increased grain production was observed when the native promoter of GIF1 was used to drive its own overexpression [23], suggesting that localized expression of GIF1 in the ovular vascular trace [23] determines grain weight.

In this study, we performed an enhancer trap screen and identified a hemizygous rice mutant hgw, which is late heading and has reduced grain width and one-thousand-grain weight. We show that HGW encodes a novel UBA-domain protein that positively regulates heading date and grain weight in rice.

Results

Isolation and Phenotypic Characterization of the hgw Mutant in Rice

In order to identify new genes that contribute to the genetic control of grain yield in rice, we screened a rice enhancer trap collection [24] for mutants with altered grain size and isolated a mutant that produced narrower and slightly longer grains than the WT control (Figure 1A–D). The final weight of 1,000 brown grains of this mutant was about 20.42±1.24 g (Figure 1E), which was approximately 22% lower than that of WT control (26.03±0.73 g; Figure 1E), indicating a significant reduction in grain weight. In addition, we found that the heading date of this mutant was about 20 days later in natural field condition compared with WT control (Figure 1F, G and H), but the numbers of panicle per plant and the numbers of grains per main panicle were not affected (data not shown). We thus designated this mutant heading and grain weight (hgw).

Grain size in rice is rigidly controlled by the volume (size) of spikelet hull [25]. Consistent with this notion, we found that the spikelet hull of hgw was markedly narrower and slightly longer than the WT control (Figure 2A). To further investigate the cause of the observed size differences, cross-sections of the central part of the spikelet hull were taken (Figure 2B–D). The cells in the spikelet hull of hgw were significantly smaller than those in the spikelet hull of WT, indicating that the reduced grain size of hgw is likely due to smaller cell sizes.

Figure 1. Phenotypic analysis of hgw. (A) and (B). Grain phenotypes of hgw and the WT control. (C). Grain width of 20 seeds of hgw and the WT control. (D). Grain length of 10 seeds of hgw and the WT control. (E). Weight of 1000 brown grains of hgw and the WT control. (F). Days to heading of hgw and the WT control. Results are presented as means ± SE (n=9). (G) and (H). Phenotypes of hgw and the WT control at maturity.

doi:10.1371/journal.pone.0034231.g001
spikelet hulls from \textit{hgw} and the WT control were imaged using scanning electron microscope (Figure 2B–D) and quantitatively compared (Figure 2E–G). We found that, in \textit{hgw} mutant, the overall circumference of outer parenchyma cell layer of lemma and palea was reduced by 23% (19% and 23% for lemma and palea, respectively) when compared to the WT control (Figure 2E), suggesting defects in cell division and/or cell elongation. Indeed, spikelet hulls of \textit{hgw} mutant contained less cells (15% and 17% less for lemma and palea, respectively) than that of the WT control (Figure 2F), and these cells showed only moderate decrease in size (4.0% and 9.8% decrease for lemma and palea, respectively) (Figure 2G). Thus, a substantial decrease in latitudinal cell number of spikelet hull resulted in the reduced grain width and grain weight of the \textit{hgw} mutant.

\textbf{Cloning of the HGW Gene}

To unravel the genetic basis of the phenotypes and changes observed in \textit{hgw} mutant, we next sought to identify the gene that was disrupted in the \textit{hgw} mutant. We isolated genomic fragments flanking the T-DNA insertion sites using thermal asymmetric interlaced (TAIL)-PCR [26] and sequenced the amplified PCR products. Blastn homology searches with the T-DNA flanking sequence against the rice whole-genome sequence (http://rice.plantbiology.msu.edu/blast.shtml) and Southern blot analysis (Figure S3) revealed that the T-DNA enhancer trap casette was inserted at a single locus on chromosome 6. By comparing the surrounding genomic sequence with the sequence of a KOME full-length cDNA clone AK121877, we found that the T-DNA insertion is located in the first exon (Figure 3A) of \textit{LO-C_Os06g06530}, a previously unreported gene with five exons. Notably, the \textit{GAL4/VP16} gene engineering in the enhancer trap cassette (Figure 3A) is in the same orientation as this interrupted gene.

To determine whether the phenotypes observed in \textit{hgw} mutants were associated with the T-DNA disruption of the \textit{LO-C_Os06g06530} gene, we genotyped T1 plants using PCR with primers (P1 and P2) flanking the insertion site and within the T-DNA enhancer trap cassette (P3, P4 and P5) (Figure 3B). We found that, while in T1 plants the presence of T-DNA insertion was detected only in plants exhibiting the mutant phenotypes described above, but not in WT-like plants (Figure 3B), PCR assays based on primers flanking the insertion site (P1 and P2) led to amplification of a genomic fragment from all the lines (Figure 3B), suggesting that the observed \textit{hgw} mutant phenotypes were caused by a hemizygous mutation and that homozygous \textit{hgw} mutant is embryonic lethal or with nonviable gametes. Consis-
tently, co-segregation of one, but not two hgw alleles with mutant phenotypes was found in T2 plants derived from independent T1 mutants, whereas neither mutant phenotypes nor T-DNA insertion was detected in the T2 progeny of the WT-like T1 plants.

To further validate whether the hgw mutant phenotypes were caused by a hemizygous mutation in the LOC_Os06g06530 loci, we performed genetic complementation experiments by introducing pC2301-6g06530, a construct carrying the LOC_Os06g06530 gene and its promoter and 3’-UTR regions into mutant callus by Agrobacterium tumefaciens-mediated transformation. The empty backbone vector pCAMBIA2301 was used as transformation control. Considering the hemizygous background of the callus used for transformation, we first performed genotyping analysis and identified 22 T1 transformants containing the T-DNA insertion, and then examined these T1 lines for both heading date and grain size phenotypes. As expected, we found that pC2301-6g06530 but not the empty backbone vector complemented the hgw mutant phenotypes. No obvious heading date difference between the rescued individuals and WT controls was observed (Figure 3C and E). The grain width of 20 seeds was 7.4±0.23 cm for the complementing lines, whereas it was 7.4±0.15 cm for the WT control (Figure 3D and F). The weight of 1000 brown grains was 23.06±1.78 g for the complementing lines, compared to 21.94±2.60 g of the WT control (Figure 3G).

Thus, a hemizygous mutation of LOC_Os06g06530 conferred the hgw mutant phenotypes in rice and we therefore renamed the LOC_Os06g06530 gene as HGW.

**HGW Encodes a Ubiquitin-Associated (UBA) Domain Protein Localized in the Cytoplasm and Nucleus**

Analysis of the full-length cDNA sequence showed that HGW encodes a novel protein with 231 amino acids. A BLASTp search against the protein sequence database at NCBI (http://www.ncbi.nlm.nih.gov) revealed no homologous genes of HGW in the rice genome and in the genomes of non-plant model organisms. Putative orthologs of HGW, however, could be identified in the indica rice cultivar (93-11) and other seed plants such as Arabidopsis, sorghum, soybean, castor bean, grapevine, papaya,
poplar and sitka spruce (Figure 4A), suggesting that the function of HGW and its orthologs is conserved during evolution of seed plants. Intriguingly, a moss ortholog of HGW was also found, indicating a role for the HGW orthologs in non-seed plants.

An extensive online database search revealed that the Arabidopsis ortholog of HGW, At5g53330, encoding a protein with a UBA domain at its carboxy terminus (http://cbsusrv04.tc.cornell.edu/users/ppdb_domain/hmmpfam.aspx?id=25787&eval=1), and more detailed protein sequence alignment analysis further showed that a UBA domain could be found at the carboxy termini of HGW and all its other orthologs examined (Figure 4A and B). The UBA domain is an approximately 40 amino acid motif that has been proposed to limit ubiquitin chain elongation and to target polyubiquitinated proteins to the 26S proteasome for degradation [27,28], indicating that HGW and its orthologs function in the ubiquitination pathways in plants. In agreement with this hypothesis, whole-genome scale co-expression analysis in rice and Arabidopsis showed that HGW and its Arabidopsis ortholog At5g53330 are co-expressed with genes encoding various components of ubiquitin-dependent sorting and degradation pathways (Table S1 and S2). More interestingly, among these components, the putative ubiquitin-conjugating enzyme Os04g57220 is homologous to At5g56150, encoding UBQUITIN-CONJUGATING ENZYME 30 (UBQ30); Whereas the putative ubiquitin family protein Os10g39620 shares high sequence similarities with At2g17190 and At2g17200, encoding two yeast homologs of polyubiquitin-biding protein DOMINANT SUPPRESSSOR OF KAR2 (Dsk2p) [29], DSK2a and DSK2b [30,31], respectively. Together, these data suggest that HGW, At5g53330 and their co-expressed genes have evolutionary conserved ubiquitination-related functions.

To learn more about HGW at the subcellular level, we created HGW-YFP and HGW-RFP fusion proteins under the control of a constitutive CaMV 35S promoter or a 2.78 kb native HGW promoter and studied the subcellular localization of HGW protein during transient expression in plant cells. Since rice protoplast cells are very small and relatively difficult to manipulate, tobacco leaf epidermal cells and onion epidermal cells were also used to facilitate the analysis of subcellular localization of HGW. Transient expression of HGW::YFP-HGW (Figure 5A, B and E), HGW::HGW-YFP (Figure 5C, D and F) and 35S::HGW-YFP (data not shown) in rice protoplasts revealed the same expression pattern for all the HGW fusions but no specific pattern that could have been suggestive of organelle (such as chloroplast, visualized by chlorophyll autofluorescence in Figure 5A and C) localization or of...
membrane localization in these cells. To determine more specifically the subcellular localization of HGW, we co-expressed HGW fusion proteins with free RFP (Figure 5B and D; and data not shown) or different fluorescent markers targeted to specific subcellular compartments and organelles (Figure S4), including Golgi markers ST-RFP [32], ST-YFP and YFP-NAG [33], endoplasmic reticulum (ER) markers ER-CFP and BIP-RFP [34] and a mitochondria marker F1-ATPase-γ:RFP [35]. We found that HGW fusion proteins co-localized with the free RFP (Figure 5B and D) but not with the subcellular fluorescent markers examined (Figure S4), indicating that HGW has a cytosolic and nuclear localization in rice cells. We further confirmed nuclear localization of HGW fusion proteins by performing the co-localization analysis with the Hoechst 33342 nuclear dye (Figure 5E and F).

Expression Levels and Pattern of HGW in WT and hgw Mutant

To further understand the role of HGW in heading date and grain width control, we examined the expression pattern of HGW in rice. Analyses of the expression data extracted from the CREP database [http://crep.ncpgr.cn/crep-cgi/home.pl] [36] suggested that HGW is expressed in all 25 tissues of Minghui 63 and Zhenshan 97 (O. sativa L. ssp. indica) at different development stage (Figure S5), including seedling and heading stages. This was further supported by RT-PCR analysis in the Zhonghua 11 (O. sativa L. ssp. japonica) background, which showed that HGW is expressed in roots and leaves in both seedling and heading stages, as well as in sheath, stem and panicles at different development stages (Figure 6A). Moreover, quantitative RT-PCR (qRT-PCR) analysis revealed that the highest transcript level of HGW was detected in the leaf, and there was a slight increase of HGW transcription at the onset of panicle development (Figure 6B).

In hgw mutant, however, qRT-PCR analysis showed that the expression level of HGW in all tissues examined, including leaf, sheath, stem and panicle, was significantly reduced compared to the WT control (Figure 6C), suggesting that the expression level of HGW is essential for the regulation of heading date and gain width in rice.

Figure 5. Subcellular localization of HGW protein during transient expression in rice protoplast cells. (A). A rice protoplast cell expressing HGW::YFP-HGW (green). Chloroplasts in the cell were visualized by chlorophyll autofluorescence (red). (B). A rice protoplast cell expressing HGW::YFP-HGW (green) and 35S::RFP (red). (C). A rice protoplast cell expressing HGW::HGW-YFP (green). Chloroplasts in the cell were visualized by chlorophyll autofluorescence (red). (D). A rice protoplast cell expressing HGW::HGW-YFP (green) and 35S::RFP (red). (E). A rice protoplast cell expressing HGW::YFP-HGW (green) and stained with the Hoechst 33342 nuclear dye (Blue). (F). A rice protoplast cell expressing HGW::HGW-YFP (green) and stained with the Hoechst 33342 nuclear dye (Blue). Nomarski DIC and merged images of the protoplasts are presented. The sizes of cells are indicated by the sizes of scale bars.

doi:10.1371/journal.pone.0034231.g005
As the GUS reporter gene in the enhancer trap cassette was in the same orientation of HGW and the T-DNA insertion was located in the first exon (Figure 3A), we reasoned that GUS expression in the hgw mutant might reflect the expression pattern of HGW in vivo and performed histological analysis of GUS activity in the mutant lines. GUS expression in the hgw mutant was detected in all tissues at the development stages examined, including ligule (Figure 7A), leaf blade (Figure 7B), sheath (Figure 7C), culm (Figure 7D and E), spikelet (lemma, palea, stamen and pistil; Figure 7F and G) and grain (Figure 7H), in line with the expression data described above. Notably, GUS staining was observed at the ovular vascular trace (Figure 7H), reminiscent of what observed for GIF1 [23], raising an intriguing possibility that HGW may function with GIF1 to control grain size and weight in rice.

**HGW Regulates Rice Heading Date and Grain Weight**

To gain insight into the molecular pathways regulated by HGW, we next asked whether HGW acts through previously identified flowering-related genes to control heading date in rice. To address this question, transcript levels of OsGI, Hd1, Ehd1, and Hd3a in leaves of 45-day-old WT and hgw mutant were compared at 11:00, 16:00 and 20:00, respectively. qRT-PCR showed no obvious differences of Ehd1 transcription in WT and hgw mutant at all the three time points examined (Figure 8A), suggesting that Ehd1 is not regulated by HGW. By contrast, transcript levels of Hd1 and Hd3a at 11:00 were reduced in hgw mutant compared with the WT control (Figure 8B and C), whereas OsGI transcription in hgw was significantly reduced at 11:00 and 16:00 (Figure 8D). These data imply that HGW differentially promotes the expression of Hd1, Hd3a and OsGI and that the regulatory interactions among different heading date-related genes are far more complex than what we have seen so far (Figure S1).

Having established the connection of HGW with known flowering-related genes, we further investigated whether HGW acts through GIF1, GW2, GW3 and GS3 to regulate grain size and weight. By analyzing the transcript levels of these genes in hgw and the WT control with qRT-PCR, we found that expression of all these genes was reduced in hgw compared with the WT control (Figure 8E). Among them, GIF1 transcription showed the most severe reduction (>90%; Figure 8E), whereas the transcript levels of other genes were decreased about 20% to 50% (Figure 8E). These data thus suggest that HGW acts as a novel upstream regulator of grain weight-related gene expression in rice (Figure S2). The co-expression of HGW with GIF1 at the ovular vascular trace indicates that HGW may function directly through GIF1 to control grain size and weight in rice.

**Discussion**

Despite enormous efforts made to date, only a few genes involved in the heading date and grain weight control in rice have been cloned and characterized. Knowledge obtained from studies of flowering-time regulation in the model plant species Arabidopsis has facilitated understanding of molecular pathways and mechanisms controlling heading date in rice, as both species adopt similar sets of molecular players to regulate flower transition. How grain weight is regulated in rice, however, remain poorly understood. It is thus essential to identify new genes that control grain weight in rice and learn about the connection between grain weight-related genes.

In this study, we demonstrate that enhancer trap screens in rice have the potential to identify novel gene functions regulating specific development processes such as heading and grain formation [24]. We provide evidence that the heading date and grain weight phenotypes observed in the hgw mutant were caused by a hemizygous mutation in the LOC_Os06g06530/HGW gene. The inability to identify homozygous null mutation in HGW could be due to that the homozygous null mutant is embryonic lethal or nonviable gametes, thus suggesting a gene dosage effect for HGW. While the mechanisms involved in the low HGW transcription level in hgw mutant remain to be elucidated, hemizygous disruption of HGW might account for low transcription levels seen in the hgw mutant, and the strong reduction in the transcription of HGW in the panicle might account for the severe phenotype observed in the panicle.

HGW encodes a novel protein with a UBA domain [27] at the carboxyl terminus, indicating that HGW functions in the ubiquitination pathway. Consistent with this indication, we found
that HGW and its Arabidopsis ortholog At5g53330 co-expressed with genes encoding various components of ubiquitin-dependent sorting and degradation pathways. Moreover, HGW localizes to the cytoplasm and nucleus, where the ubiquitin-proteasome system resides [37]. Computational prediction of subcellular localization of co-expressed gene products listed in Table S1 and S2 further suggest that HGW interacts with most of these ubiquitination components in the cytoplasm, where they might act together to regulate heading date and grain weight in rice. Intriguingly, among the four grain-weight related genes identified previously through map-based cloning of QTLs, three of them encoding proteins that are possibly associated with the ubiquitination machineries. GW2 has been shown to encode a cytosolic RING-type protein with E3 ubiquitin ligase activity [20] and GW5/qSW5 encodes a nuclear protein which physically interacts with a polyubiquitin [21,22], whereas the protein encoded by GS3 contains a putative cysteine-rich domain of the tumour necrosis factor receptor (TNFR), which likely colocalizes with ubiquitin in human cells [38]. These data together raise the possibility that HGW, GW2, GW5/qSW5 and GS3 act through the same ubiquitination pathways to determine grain size and grain weight in rice. In agreement with this hypothesis, our qRT-PCR revealed that HGW promotes, at the transcriptional level, the mRNA expression of GW2, GW5/qSW5, GS3 and GIF1. The strong reduction of GIF1 transcription in the hgw mutant background further implies that HGW acts directly upstream of GIF1 to regulate the activity of cell-wall invertase during the early formative stage of rice grain/endosperm development.

Ubiquitination has also been found to play a critical role in the photoperiodic control of flowering time in Arabidopsis. Mutations in the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) lead to extreme early flowering under SD [39], indicating that COP1 regulates the 26S proteasome-mediated degradation of flowering time-related factors. Indeed,

Figure 7. Expression pattern of HGW indicated by GUS staining in different tissues of the hgw mutant. GUS staining was observed in ligule (A), leaf blade (B), sheath (C), culm (D and E), spikelet (F and G) and grains (H). (E) shows GUS staining in the cross-section of culm, and (G) reveals GUS staining in stamen and pistil.

doi:10.1371/journal.pone.0034231.g007
two recent reports showed that COP1 contributes to day length perception by reducing the abundance of CO during the night and thereby delaying flowering under SD [40,41]. Moreover, COP1 has been shown to interact with EARLY FLOWERING 3 (ELF3), which allows COP1 to interact with GI, leading to targeted destabilization of GI [42]. Together, these data explain why the GI-CO-FT pathway is active only during LD in Arabidopsis, and destabilization of GI [42].

The expression levels of examined genes were normalized to the UBQ1 expression levels. All values are based on at least three biological and three technical repeats and presented as means ± SE (n=3).

doi:10.1371/journal.pone.0034231.g008

Figure 8. qRT-PCR expression analysis of heading- and grain weight-related genes in WT and hgw mutant. (A) to (D). Expression of heading-related genes including Ehd1 (A), Hd1 (B), Hd3a (C) and OsGI (D) in panicles collected from 3 time points. 1: 11:00, 2: 16:00 and 3: 20:00. (E). Expression of grain weight-related genes including GIF1, GW2, GW5 and GS3 in panicles of WT and hgw mutant. The transcript levels of examined genes were normalized to the UBQ1 expression levels. All values are based on at least three biological and three technical repeats and presented as means ± SE (n=3).

Materials and Methods

Plant Materials and Growth Conditions
The hgw mutant was identified from a screen with the rice T-DNA insertion lines generated with an enhancer trap system [24]. Both the hgw mutant and the WT control used in this study are in the Zhonghua 11 (O. sativa L. ssp. japonica) background. All the rice lines were planted under natural growth conditions in the rice-growing seasons at the experimental field of Huazhong Agricultural University, except for the plant materials used for the expression analysis of heading date-related genes, which were obtained from plants grown in a SD condition in the greenhouse (about 10 h light/14 h darkness, 28°C).

Isolation of Flanking Sequence and Genotyping
DNA extraction and flanking sequence isolation were performed as described previously [26]. The rice genome sequence corresponding to the T-DNA flanking sequence was identified with BLASTN (http://rice.plantbiology.msu.edu/blast.shtml). The co-segregation relationship between the phenotype and the T-DNA insertion was analyzed by two sets of PCR, one using the gene-specific primer pair and the other using a gene-specific primer and a T-DNA border primer. PCR was performed with the following cycling profile: 94°C for 5 min, followed by 30 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, and a final 7-min extension at 72°C. Primers for genotyping are listed in Table S3.

Southern Blot Analysis
Southern blot analysis was performed essentially as previously described [24]. Genomic DNA isolated from T0 transgenic rice plants was digested with HindIII and transferred to a Hybond N+ nylon membrane for Southern blot analysis. P1 and P5 primers (see Figure 3A and Table S3) designed based on the coding
sequence in the region of GAL4/VP16 were used to generate the GAL4/VP16-specific probe for Southern blot hybridization.

**Sequence and Phylogenetic Analyses**

HGW protein sequence was translated from a KOME full-length cDNA sequence (http://cdna01.dna.afirc.go.jp/cDNA) [45]. Protein sequences of HGW homologs from the other plant species were obtained by using blast search against the NCBI database (http://www.ncbi.nlm.nih.gov). The number and position of exons and introns were derived from the Eutrex Gene database (http://www.ncbi.nlm.nih.gov/gene/) by comparison of the cDNAs with their corresponding genomic DNA sequences. Multiple protein sequences alignment was performed with ClustalX Version 2.0 [46], and the result was refined manually.

**Expression Analysis with RT-PCR and qRT-PCR**

Total RNA of various tissues from Zhonghua 11 (O. sativa L. ssp. japonica), hgw mutant and WT control were extracted with Trizol reagent (Invitrogen) according the manufacturer’s instructions. PCR amplifications were performed as following cycling profile: 94°C for 5 min; 28–35 cycles at 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min; and 72°C for 7 min. Primers for RT-PCR experiments are listed in Table S3. Each experiment was repeated twice, and the rice GAPDH gene was used as an internal control.

PRT-PCR reactions were carried out on the bio-rad cfx96 real-time PCR system using three-step cycling conditions of 95°C for 2 min, followed by 40 cycles of 95°C for 20 s, 56°C for 20 s, and 72°C for 30 s. At the end of each experiment, a melting curve was determined for each primer pair at a temperature stage from 72°C to 95°C to check the specificity of annealing. Primers targeting ubiquitin were used to normalize the expression data for each gene. The rice UBQUITIN EXTENSION PROTEIN 1 (UBQ1; Os05g13170) was used as reference for qRT-PCR analyses. At least three technical replicates were performed for each biological replicate. The primers were listed in Table S3.

**Co-expression Analysis**

Co-expression analysis of Os06g06530 was performed by searching against the rice oligonucleotide array database at http://www.ricearrary.org/coexpression/coexpression.shtml, with a correlation coefficient cutoff set at 0.5. To get the co-expressing genes of At1g33330, another web-tool, GeneCAT [47] for Arabidopsis thaliana (http://genecat.mpq.de/~36706/genepl.html) was used according to the developer’s instruction, with r-value cutoff set as 0.5.

**Vector Construction and Rice Transformation**

A 7.5-kb XhoI genomic fragment isolated from the Nipponbare BAC clone OSJNBA003J10 was subcloned into the binary vector pCAMBIA2301 digested with SalI as the complementary vector, giving pC2301-6g06530. The empty vector pCAMBIA2301 was used as a control, giving pC2301. The callus culture induced from the cDNAs with their corresponding genomic DNA sequences.

**Subcellular Localization Analysis**

For subcellular localization analysis in rice protoplast cells, the sterilized Nipponbare seeds were grown on half-strength MS medium at 28°C for 14 days in dark. Leaf and stem tissue was cut into approximately 0.5 mm strips using very sharp razors. The protoplast isolation and DNA transfection were performed as described by [46].

For subcellular localization analysis in tobacco leaf epidermal cells, N. tabacum sp. plants were grown in the plant room at 22°C with 16 h light/8 h dark for 4–6 weeks prior to Agrobacterium infiltration. Agrobacterium-mediated infiltration of tobacco leaf epidermal cells was performed as previously described [49]. Briefly, Lower leaves of N. tabacum sp. plants were infiltrated with the diluted bacteria using a syringe. For co-expression the bacteria were mixed in appropriate volumes of infiltration buffer prior to injection into the leaves. 5-bromo-4-chloro-3-indolyl-β-D-glucuronidase (GUS) Activity

Expression of GUS in rice tissues was assayed essentially as previously described [24]. Different rice tissues were cut off from the plants, transferred to microfuge tubes, where they were submerged into the GUS staining solution (50 mM sodium phosphate at PH 7.0, 0 mM EDTA, 0.1% Triton X-100, 1 mg ml⁻¹ X-Gluc, 100 μg ml⁻¹ chloramphenicol, 1 mM potassium ferriyanide, 1 mM potassium ferrocyanide, and 20% methanol), followed by vacuum infiltration for 20 minutes, and then incubated at 37°C for about 24 hours. The stained samples were finally transferred to another tube and fixed with 70% ethanol.

**Scanning Electron Microscopy and Cross-section Analysis of Spikelet Hulls**

For scanning electron microscopy, samples were prepared according to a previously reported method [50] with some modifications. In brief, rice tissues were excised with a blade and immediately placed in mixture of 70% ethanol, 5% acetic acid, and 3.7% formaldehyde for 24 h. Samples were then critical-point dried, sputter-coated with gold, and observed with a scanning electron microscope (S570; Hitachi, Tokyo, Japan).

Cross-section images from scanning electron microscopy were used for phenotypic analysis of spikelet hulls. The total length (circumference), cell number and mean cell length in the outer parenchymal cell layers of spikelet were analyzed according to Song et al. 2007 [20] with ImageJ (version 1.44).

**Supporting Information**

Figure S1 A summary diagram of the regulatory interactions between genes involved in heading date
control in rice. SD: short-day condition. LD: long-day condition.

(TIF)

Figure S2 A summary diagram of the regulatory interactions between genes involved in grain weight control in rice.

(TIF)

Figure S3 Southern blot analysis in T0 hgw plant revealed a single T-DNA insertion in its genome. (A). A schematic diagram of the T-DNA region in the pFX-E24.2-15R vector used for generation of enhancer trap rice lines [24]. The right border (RB) and left border (LB) regions of the T-DNA are indicated. GAL4/VP16, a gene generated by fusing yeast transcriptional activator GAL4 DNA-binding domain with the Herpes simplex virus VP16 activation domain; GUS/Plus, a modified β-glucuronidase; 6×UAS, upstream activator sequence with six repeats; Ḥph, hygromycin phosphotransferase; Amp', ampicillin-resistance gene. H, HindIII site. (B). Southern blot hybridization of T0 enhancer trap transformants. The arrow points to the T0 hgw mutant (lane 5), and the rest lanes stand for other T0 enhancer trap transformants examined. Genomic DNA from the T0 enhancer trap transformants was digested with HindIII and hybridized with a GAL4/VP16-specific probe.

(TIF)

Figure S4 Subcellular localization of HGW protein during transient expression in plant cells. (A). Tobacco leaf epidermal cells expressing 35S promoter driven HGW-RFP (red) and ST-YFP (green, top panel), YFP-NAG (green, middle panel) or ER-CFP (green, bottom panel). (B). Onion epidermal cells expressing 35S promoter driven HGW-YFP (green) and ST-RFP (red, top panel), Fl-ATPase-γ-RFP (red, middle panel) or BIP-RFP (red, bottom panel). The sizes of cells are indicated by the sizes of scale bars.

(TIF)

Figure S5 HGW expression in Minghui 63 and Zhenshan 97 (O. sativa L. ssp. indica) at different development stages. Tissues examined: (1) seed at 72 h after imbibition; (2) calli at 15 days after subculture; (3) embryo and radicle after germination; (4) leaf and root at three-leaf stage; (5) root at seedling with two tillers; (6) shoot at seedling with two tillers; (7) leaf at young panicle of secondary branch primordium differentiation stage; (8) sheath at young panicle of secondary branch primordium differentiation stage; (9) young panicle of secondary branch primordium differentiation stage; (10) young panicle at pistil/stamen primordium differentiation stage; (11) young panicle at pollen-mother cell formation stage; (12) leaf at 4–5 cm young panicle stage; (13) sheath at 4–5 cm young panicle stage; (14) panicle at 4–5 cm young panicle stage; (15) flag leaf at 5 days before heading; (16) culm at 5 days before heading stage; (17) panicle at heading stage; (18) culm at heading stage; (19) hull at 1 day before flowering stage; (20) stamen at 1 day before flowering stage; (21) spikelet at 3 days after pollination stage; (22) endosperm at 7 days after pollination stage; (23) flag leaf at 14 days after heading stage; (24) endosperm at 14 days after pollination stage; (25) endosperm at 21 days after pollination stage. Signal value represents expression level. The error bars are obtained from two replications.

(TIF)

Table S1 Co-expression analysis of HGW (selected gene list).

(PDF)

Table S2 Co-expression analysis of At5g53350 (selected gene list).

(PDF)

Table S3 Primers for genotyping and expression analysis.

(PDF)

Acknowledgments

We thank Inhwan Hwang for the mitochondria marker F1-ATPase-γ-RFP and the Golgi marker starch ST-RFP, YiZhu Zhou for the endoplasmic reticulum marker p-BIP-RFP, Rod Wing for the Nipponbare BAC clone OSJN-Ba0033J10, Jianbo Cao and LiHong Qin for technical assistance with the scanning electron microscope, and Henghao Xu for help in qRT-PCR analysis.

Author Contributions

Conceived and designed the experiments: JL HWC QFZ JX. Performed the experiments: JL HWC. Analyzed the data: JL HWC QFZ JX. Contributed reagents/materials/analysis tools: VHZ TMM CYW. Wrote the paper: JL JX.

References

1. Zhang Q (2007) Strategies for developing Green Super Rice. Proc Natl Acad Sci U S A 104: 16402–16409.
2. Zhang Q, Li J, Xue Y, Han B, Ding XW (2008) Rice 2020: A Call For An International Coordinated Effort In Rice Functional Genomics. Mol Plant 1: 715–719.
3. Xing Y, Zhang Q (2010) Genetic and Molecular Basis of Rice Yield. Annual Review of Plant Biology 61.
4. Tsugi H, Taoka KI, Shimamoto K (2010) Regulation of flowering in rice: two florigen genes, a complex gene network, and natural variation.Curr Opin Plant Biol.
5. Yano M, Katayose Y, Ashikari M, Yamanouchi U, Mouna L, et al. (2000) Hd1, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene CONSTANS. Plant Cell 12: 2473–2484.
6. Izawa T, Ohkawa T, Sugiyama N, Tamai K, Yano M, et al. (2002) Phytocrome mediates the external light signal to repress FT orthology in photo period flowering of rice. Genes Dev 16: 2006–2020.
7. Izawa T (2007) Adaptation of flowering-time by natural and artificial selection in Arabidopsis and rice. J Exp Bot 58: 3091–3097.
8. Kujima S, Takahashi Y, Kobayashi Y, Mouna L, Sasaki T, et al. (2002) Hd3α, a rice ortholog of the Arabidopsis FT gene, promotes transition to flowering downstream of Hd1 under short-day conditions. Plant Cell Physiol 43: 1096–1105.
9. Tamaki S, Matsumo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3α protein is a mobile flowering signal in rice. Science 316: 1033–1036.
10. Komiya R, Regami A, Tamaki S, Yokoi S, Shimamoto K (2008) Hd3α and RFT1 are essential for flowering in rice. Development 135: 767–774.
11. Hayama R, Yokoi S, Tamaki S, Yano M, Shimagomoto K (2003) Adaptation of pho toperiodic control pathways produces short-day flowering in rice. Nature 422: 719–722.
12. Tseng TS, Salome PA, McChung CR, Olezowski NE (2004) SPINDLY and GIGANTEA interact and act in Arabidopsis thaliana pathways involved in light responses, flowering, and rhythms in cotyledon movements. Plant Cell 16: 1550–1563.
13. Doi K, Izawa T, Fuse T, Yamanouchi U, Kubo T, et al. (2004) Ehd1, a B-type response regulator in rice, confers short-day promotion of flowering and controls FT-like gene expression independently of Hd1. Genes Dev 18: 926–936.
14. Xue W, Xing Y, Weng X, Zhao Y, Tang W, et al. (2008) Natural variation in Ghd7 is an important regulator of heading date and yield potential in rice. Nat Genet 40: 761–767.
15. Wu C, You C, Li C, Long T, Chen G, et al. (2008) RD11, encoding a Cys2/His2-type zinc finger transcription factor, acts as a master switch from vegetative to floral development in rice. Proc Natl Acad Sci U S A 105: 12915–12920.
16. Matsuoka K, Yamanouchi U, Wang XZ, Minobe Y, Izawa T, et al. (2008) Ehd2, a rice ortholog of the maize INDETERMINATE1 gene, promotes flowering by up-regulating Ehd1. Plant Physiol 148: 1425–1435.
17. Colasanti J, Yuan Z, Sundaresan V (1998) The indeterminate gene encodes a zinc finger protein and regulates a leaf-generated signal required for the transition to flowering in maize. Cell 93: 593–603.
18. Fan C, Xing Y, Mao H, Lu T, Han B, et al. (2006) G53, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. Theor Appl Genet 112: 1164–1171.
Motivated by the need for understanding the fundamental aspects of intracellular trafficking, researchers have delved into the role of ADP-ribosylation factors (ARFs) in the Arabidopsis thaliana plant. ARFs are small GTPases that play key roles in membrane trafficking and vesicle formation. They are essential for the proper function of the Golgi apparatus and endosomal systems, facilitating the transport of materials between different cellular compartments.

One study by Jin et al. (2001) highlighted the importance of ARFs in regulating intracellular trafficking. They demonstrated that deletion of a gene associated with grain size increased yield during rice domestication, with a potential role for ARFs in this process. This work underscores the significance of ARFs in shaping plant architecture and yield potential.

Another study by Xu and Scheres (2005) focused on the dissection of Arabidopsis ADP-ribosylation factor 1 (ARF1) function in epidermal cell polarity. This research provided insights into how ARFs can influence the polarization of plant cells, which is crucial for the development of plant structures.

A recent work by Wu et al. (2003) involved the development of enhancer trap lines for functional analysis of the rice genome. This approach has been instrumental in elucidating the roles of various genes in plant development, including those involved in cell polarity and trafficking.

These findings, along with others, have contributed to a better understanding of the complex interactions between ARFs and other cellular components, leading to advancements in our knowledge of plant biology. The integration of ARF function with other cellular processes, such as ubiquitination and the proteasome, is a critical area of ongoing research to fully comprehend the regulatory mechanisms in plant cells.