Rice

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Gγ-protein GS3 Function in Tight Genetic Relation with OsmiR396/GS2 to Regulate Grain Size in Rice

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Abstract

Manipulating grain size demonstrates great potential for yield promotion in cereals since it is tightly associated with grain weight. Several pathways modulating grain size have been elaborated in rice, but possible crosstalk between the ingredients is rarely studied. OsmiR396 negatively regulates grain size through targeting OsGRF4 (GS2) and OsGRF8, and proves to be multi-functioning. Here we showed that expression of *GS3* gene, a Gγ-protein encoding gene, that negatively regulates grain size, was greatly down-regulated in the young embryos of MIM396, GRF8OE and GS2OE plants, indicating possible regulation of *GS3* gene by OsmiR396/GRF module. Meanwhile, multiple biochemical assays proved possible transcriptional regulation of OsGRF4 and OsGRF8 proteins on *GS3* gene. Further genetic relation analysis revealed tight genetic association between not only OsmiR396 and *GS3* gene, but also *GS2* and *GS3* gene. Moreover, we revealed possible regulation of GS2 on four other grain size-regulating G protein encoding genes. Thus, the OsmiR396 pathway and the G protein pathway cross talks to regulate grain size. Therefore, we established a bridge linking the miRNA-transcription factors pathway and the G-protein signaling pathway that regulates grain size in rice.

Keywords Grain size, OsmiR396, OsGRF, G Protein, *GS3*

Introduction

Rice is one of the main crops in the world which provides food for more than one half of the world population. The demand for rice yield is likely to increase approximately 1.5 times by the year 2050 (Haque et al. 2015). To meet such a global demand, strategies to increase rice productivity have always been the main concern. Three components contribute to yield in rice, such as the number of panicles, the number of grains per panicle, and grain weight, which are tightly associated with the number of tillers, panicle architecture, and grain size respectively (Xing and Zhang 2010). The number of panicles is in tight association with plant architecture, which is under regulation of miRNAs, transcriptional factors and other interacting factors in association with hormone homeostasis or regulation (Guo T et al. 2020; Guo W et al. 2020). Among them, tiller number is an important trait that closely related to yield due to their potential to bear panicles. Ideal plant architecture (IPA) includes low tiller numbers with few unproductive tillers, more grains per panicle, and thick and sturdy stems (Jiao et al. 2010). Over expression of OsmiR156 tremendously promoted the number of tillers, however, the overall shortened

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architecture also influences the length of the panicle which attenuate its possibility in yield promotion (Dai et al. 2018). Rice panicle architecture determines the grain number per panicle and therefore impacts grain yield. *RGN1* (REGULATOR OF GRAIN NUMBER1) regulates lateral grain formation to control grain number and shape panicle architecture (Li et al. 2022). The OsER1-OsMKKK10-OsMKK4-OsMPK6 pathway shapes panicle architecture by regulating cytokinin metabolism (Guo T et al. 2020). Further study reveals that a small peptide family synergistically regulate rice panicle morphogenesis through interacting with OsER1 and activating the mitogen-activated protein kinase (MAPK) cascade, which is also a major pathway regulating grain size (Li et al. 2019; Guo et al. 2023). OsmiR396 proves to be multi-functional, with down-regulation of OsmiR396 modulating auxiliary branches, spikelets and grain size through regulating *Growth Regulating Factor 4* (*OsGRF4*), *OsGRF6*, and *OsGRF8*, respectively (Gao et al. 2015; Zhang et al. 2020).

Manipulating grain size demonstrates great potential for yield promotion in cereals since it is tightly associated with grain weight. Genetically, grain size is under complicated molecular and genetic modulation of different signaling and metabolic pathways. Due to the significance of grain size for supporting world population and in evolution, great attention has been paid to the molecular basis for grain size regulation. Accordingly, quite a few grain-size-determining quantitative trait loci (QTLs) have been identified, and several grain-size-regulating signaling pathways have been formed, such as ubiquitin-proteasome degradation, MAPK signaling, Heterotrimeric GTP-binding regulatory proteins (G protein) signaling, phytohormone signaling, and transcriptional regulation pathway (Li et al. 2019).

G proteins consisting of Gα, Gβ and Gγ are key regulators of a multitude of signaling pathways in both animals and plants, which mediate transmembrane signaling by coupling to the cell surface–localized G Protein-Coupled Receptor (GPCRs) (Liang et al. 2018). Different from animal, plants do not have functional GPCRs, and formed unique mechanisms of G protein signaling that plays essential roles in both development and response to biotic and abiotic stresses, with great attention being paid to its potential to yield promotion (Pandey 2019). In rice, seven G proteins have been identified to regulate grain size, they are RGA1(α), RGB1(β), and five γ-subunit proteins, GS3, OsDEP1, GGC2, RGG1, and RGG2 (Ueguchi-Tanaka et al. 2000; Utsunomiya et al. 2011; Botella 2012; Liu et al. 2018; Sun et al. 2018; Miao et al. 2019; Tao et al. 2020). Mutation of *RGA1* greatly reduced grain size (Fujisawa et al. 1999). DEP1 and GGC2 positively regulate grain size in combination with RGB1, and additive effect exists between DEP1 and GGC2, while GS3 reduces grain length by competitively interacting with RGB1 (Sun et al. 2018). Both RGG1 and RGG2 negatively regulate grain size (Miao et al. 2019; Tao et al. 2020). Furthermore, G proteins prove to be multifunctional. For example, DEP1 associates with RGA1 and RGB1 to mediate nitrogen response, and modulation of G protein activity might provide environmentally sustainable increases in yield (Sun et al. 2014). Besides, DEP1 regulates plant architecture including panicle architecture (Huang et al. 2009) and grain quality (Huang et al. 2022). In addition to grain size regulation, the Gγ-subunit protein GS3 is identified as a heat tolerance locus, *THEROMOTOLERANCE 2 (TT2)*, which function through regulating wax synthesis (Kan et al. 2022). Moreover, an *Alkaline Tolerance 1* (*AT1*) locus identified in sorghum is homologous to *GS3*, which improves productivity in several crop species under alkaline tolerance conditions (Zhang et al. 2023).

microRNA (miRNA) is increasingly being realized as a powerful factor for breeding due to their essential roles in various developmental processes including tiller number, grain size, and panicle branching (Tang and Chu 2017). Meanwhile, miRNAs also function in response to various stresses (Khraiwesh et al. 2012; Ren et al. 2023). Moreover, targets of miRNA are mostly transcriptional factors (Llave et al. 2002), which are essential functional mediators of miRNAs through forming the repressor miRNA/target module. The typical *IPA* gene is one target of OsmiR156 (Jiao et al. 2010), in accordance, over expression of the target mimicry (MIM) OsmiR156 improved grain size (Franco-Zorrilla et al. 2007; Dai et al. 2018). The elongation of palea and lemma implies grain enlarging potential, and correspondingly, over expression of the targets of OsmiR172, *AP2* genes, obviously decreased grain size (Dai et al. 2016). OsmiR167 negatively, while its target OsARF12, positively regulate grain size, downstream of OsmiR159 (Qiao et al. 2021; Zhao et al. 2023), which positively regulated grain size (Zhao et al. 2017; Gao et al. 2018). Over expression of OsmiR397 increases grain size (Zhang et al. 2013), while down regulation of OsmiR398 through short tandem target mimics (STTM) decreases grain size (Yan et al. 2012; Zhang et al. 2017). Besides regulating panicle architecture (Gao et al. 2015), quite a few studies indicate that OsmiR396 might play a pivotal role in determining grain size (Liebsch and Palatnik 2020). One target of OsmiR396, *OsGRF4*, has been respectively identified as several grain-size QTLs including *GS2* (Duan et al. 2015; Hu et al. 2015). Another target, *OsGRF8*, also positively regulates grain size, and OsmiR396/OsGRF8 modulates grain size through regulating OsmiR408, knock out of which results in smaller grains (Yang et al. 2021). Moreover, the regulation of OsmiR396 on grain size is conserved in wheat (Yu et al. 2022).

Due to the complicated genetic and regulation factors involved in grain size control, the crosstalk among different grain size factors is yet to be elucidated to further our understanding of the underlying interweaved mechanism and guide modern crop breeding. Specifically, if *GS3* acts downstream of other factors such as miRNAs to regulated grain size remains to be unrevealed. Here, through expression analysis, biochemical assays, and genetic relation analysis between OsmiR396 and *GS3*, and *GS2* and *GS3*, we revealed that tight genetic interaction exists between OsmiR396/OsGRF module and *GS3* gene. Furthermore, the high possibility of GS2 modulating other grain size-regulating G proteins was explored. Therefore, we proved that the miRNA-transcriptional regulatory factors pathway and the G-protein signaling pathway, two typical pathways regulating grain size, could cross talk.

Results

OsGRF8 Might Regulate *GS3* Gene at the Transcriptional Level

Increasing evidence suggests that OsmiR396 play pivotal roles in grain size regulation in rice (Zhang et al. 2020; Yang et al. 2021). To investigate if the OsmiR396/OsGRF module could cross talk with factors in other signaling pathways regulating grain size, we first checked the expression of some grain size-regulating genes in 5 days-after-fertilization (DAFs) of MIM396 (overexpressing mimicry OsmiR396) plants and ZH11 plants (Fig. 1A) (Yang et al. 2021). Clearly, most of these genes were influenced in MIM396 plants as compared with in wild type (WT) ZH11 plants. Among them, the *GS3* gene, which negatively regulated grain size, was greatly down-regulated (Fig. 1A). We further isolated the 4 DAFs and 5 DAFs embryos of MIM396, and GRF8OE plants, both of which showed promoted grain size (Yang et al. 2021). It was confirmed that *GS3* gene was obviously down-regulated in 4 DAFs and 5 DAFs embryos of both the MIM396 and GRF8OE plants, as compared with in those of WT ZH11 plants (Fig. 1B).

We thus wonder if OsGRF8 could regulate *GS3* gene transcriptionally. To this end, we analyzed the promoter of *GS3*. The binding motif of GRF proteins is CGC(G)A(C)G(A) (Gao et al. 2015), it was revealed that there are 21 putative GRF binding motifs in the 1.8 Kb *GS3* promoter upstream of ATG start code (Fig. 1C), indicating possible binding of OsGRF to them. To verify this possibility, we carried out electrophoretic mobility shift assay (EMSA) using the DNA binding WRC (Trp, Arg, Cys) domain, which binds DNA (Kim et al. 2003), of OsGRF8 protein and revealed that it indeed bound to the *GS3* promoter fragments (Fig. 1D). In the chromatin immunoprecipitation (ChIP) assay, the GRF8-fused GFP protein bound more fragments in the motif-containing region of *GS3* promoter in GRF8OE plants (fused with GFP, Yang et al. 2021) (Fig. 1E). Further in a Dual-LUC assay, OsGRF8 protein inhibited *GS3* expression and resulted in a decreased LUC/REN value than the Flag control (Fig. 1F). Thus, these biochemical assays collectively indicated that OsGRF8 protein might directly bind to the promoter of *GS3* gene and represses its expression.

Genetic Interaction Between OsmiR396 and *GS3* Gene

Next, we investigated the possible genetic interaction between the OsmiR396/OsGRF module and *GS3* gene. The MIM396 plants show elongated and enlarged grains (Yang et al. 2021), and *GS3* negatively regulates grain size (Sun et al. 2018). We crossed the MIM396 plants to the GS3-4OE plants, in which only the N-terminal organ size regulation (OSR) domain was over expressed and the negative effect of *GS3* on grain size was further enhanced (Sun et al. 2018). In the cross plants, the genomic level (Fig. S1A) and mRNA level (Fig. 2A) of the *IPS* backbone of mimicry OsmiR396 used in the construction of MIM396 plants were confirmed (Yang et al. 2021). Accordingly, as an example, the miR396d was down-regulated (Fig. S1B). Meanwhile, in the cross plants, the *GS3* gene (the OSR domain) was up-regulated as in the GS3-4OE plants (Fig. 2B). These data collectively confirmed the successful cross of MIM396 plants to the GS3-43OE plants. As a result, the cross plants showed a small grain size similar to that of GS3-4OE (Fig. 2C), with the 1000-grain-weight much lower than that of the MIM396 plants (Fig. 2D).

Meanwhile, we crossed the GS3-4OE plants to the MIM396 plants. In the positive cross plants, the *IPS* gene was clearly up-regulated as in the MIM396 plants, and as in a negative cross plant (Fig. 3A). At the same time, the OSR domain of the *GS3* gene was up-regulated in the positive cross plants as in the GS3-4OE plants (Fig. 3B). As a result, the grain size of the positive cross plants, but not the negative ones, was similar to that of the GS3-4OE plants (Fig. 3C), accordingly, the 1000-grain-weight of the positive cross plants was much similar to that of GS3-4OE (Fig. 3D). Therefore, the reciprocal cross tests between the MIM396 plants and the GS3-4OE plants revealed tight genetic association between OsmiR396 and *GS3* gene.

Genetic Interaction Between *GS2* and *GS3* Gene

miRNAs fulfill their function through negative regulation of their target genes, which usually encode transcription factors. *OsGRF8* is one target of OsmiR396 that positively regulates grain size (Yang et al. 2021). When we crossed the GRF8OE plants to the GS3-4OE plants, co-suppression might be brought about (Fig. S2A, B). Since *GS2* is also one target of OsmiR396 that function essentially in grain size regulation (Duan et al. 2015; Hu et al. 2015), and furthermore, the WRC domains of the

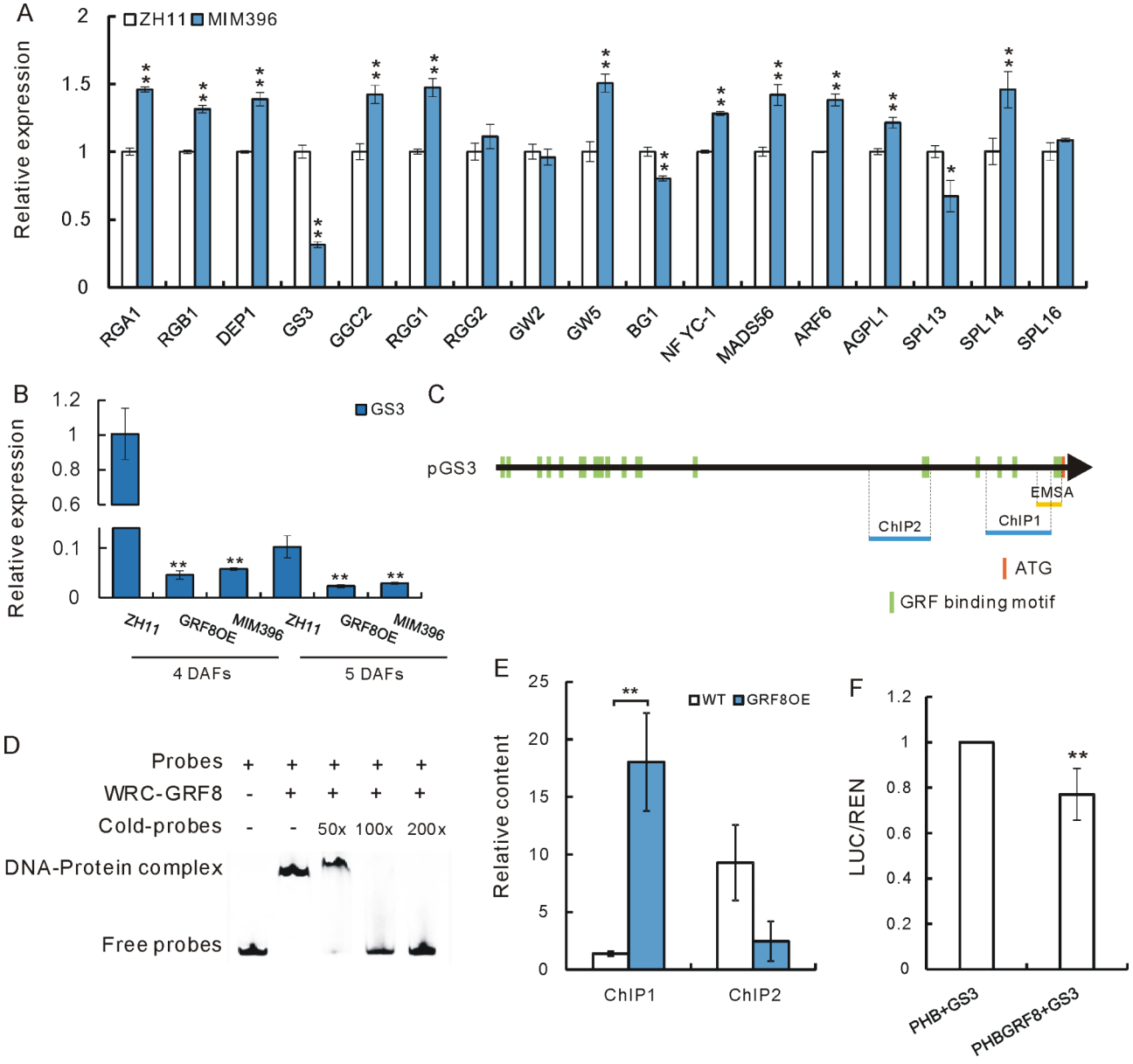

Fig. 1 Detection of transcriptional regulation of OsGRF8 on *GS3*. **A**, Expression of a few grain size regulating genes in the young embryos of 5 DAFs of the MIM396 and WT ZH11 plants. Data are means ± SD ($n = 3$). **B**, Expression of *GS3* gene in the young embryos (4 DAFs and 5 DAFs respectively) of the MIM396, GRF8OE and WT ZH11 plants. Data are means ± SD ($n = 3$). Asterisks in (**A**) and (**B**) indicate significant differences with ZH11 respectively as determined by the Student's *t*-test (**, $P < 0.01$). **C**, The sketch map of the 1.8 Kb promoter of the *GS3* gene. Black arrow in the middle indicated the *GS3* gene with transcription direction. The green bars indicated the putative GRF binding motifs. The red bar indicated the ATG start code. The yellow horizontal line indicated the EMSA fragment used in (**D**) with corresponding size and site on the promoter. The blue horizontal lines indicated the ChIP fragments used in (**E**) with corresponding size and site on the promoter. **D**, EMSA of the WRC domain of OsGRF8 protein binding on the *GS3* promoter fragments. **E**, ChIP analysis of the GRF8OE plants (fused with GFP) and WT plants using anti-GRF antibody. Data are means ± SD ($n = 3$). Asterisks indicate significant differences with WT as determined by the Student's *t*-test (**, $P < 0.01$). **F**, The LUC/REN ratio of the Dual-LUC assay of the OsGRF8 protein and the *GS3* promoter. Data are means ± SD ($n = 3$). Asterisks indicate significant differences compared with "PHB + GS3" as determined by the Student's *t*-test (**, $P < 0.01$)

OsGRF1, OsGRF4, and OsGRF8 proteins show very high similarity (Fig. S3). We thus detected if GS2 could also regulate *GS3* transcriptionally. In a Dual-LUC assay, GS2 protein inhibited *GS3* expression (Fig. 4A) and resulted in a decreased LUC/REN value than the Flag control (Fig. 4B). Furthermore, in EMSA assay, the WRC domain of GS2 protein bound to the *GS3* promoter fragments (Fig. 4C). Therefore, GS2 protein might also bind to the *GS3* promoter and inhibit its expression.

To detect the genetic interaction between *GS2* and *GS3* gene, we crossed the GS3-4OE plants to the GS2OE plants, simultaneous up-regulation of *GS2* genes and the

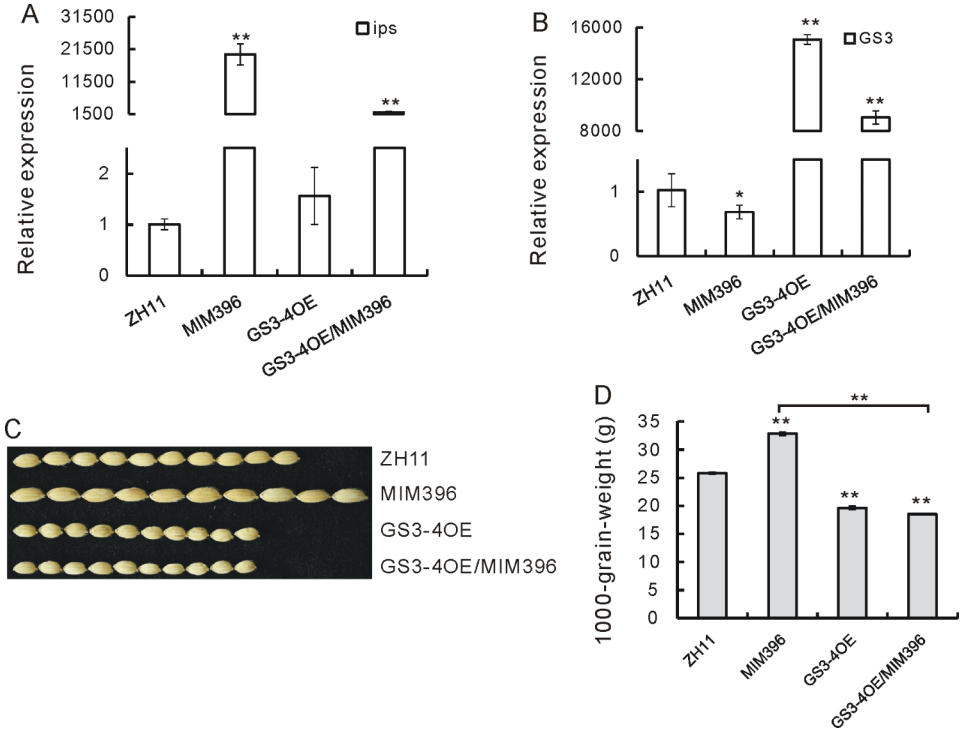

Fig. 2 Molecular detection and grain phenotype of the cross between the MIM396 and GS3-4OE plants. **A**, Expression of the *IPS* backbone in ZH11, MIM396, GS3-4OE and the GS3-4OE/MIM396 plants. Data are means ± SD ($n = 3$). **B**, Expression of the *GS3* gene in ZH11, MIM396, GS3-4OE and the GS3-4OE/MIM396 plants. Data are means ± SD ($n = 3$). Asterisks in (**A**) and (**B**) indicate significant differences with ZH11 as determined by the Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$). **C**, Picture of 10 grains of ZH11, MIM396, GS3-4OE and the GS3-4OE/MIM396 plants respectively. **D**, 1000-grain-weight of ZH11, MIM396, GS3-4OE and the GS3-4OE/MIM396 plants. Data are means ± SD ($n = 10$). Asterisks indicate significant differences with ZH11 or as indicated as determined by the Student's *t*-test (**, $P < 0.01$)

OSR domain of *GS3* gene was detected in the cross plants (Fig. 5A, B). Grain size of the cross plants were similar to that of the WT, smaller than that of the GS2OE plants while bigger than that of the GS3-4OE plants (Fig. 5C). Accordingly, the 1000-grain-weight of the cross plants was higher than that of the GS3-4OE plants, while lower than that of the MIM396 plants, and much similar to that of the WT ZH11 plants (Fig. 5D).

Possible Regulation of GS2 on the Other Grain Size-regulating G Proteins

Now that there are tight genetic interaction between OsmiR396/GS2 pathway and the *GS3* gene, we wonder that other grain size-regulating G proteins might also genetically associated with the OsmiR396/GS2 module. There are seven G proteins regulating grain size in rice (Ueguchi-Tanaka et al. 2000; Utsunomiya et al. 2011; Botella 2012; Liu et al. 2018; Sun et al. 2018; Miao et al. 2019; Tao et al. 2020), we firstly respectively checked their expression in the young panicles of the MIM396 and the GS2OE plants, it was revealed that other four G protein encoding genes, *RGA1*, *DEP1*, *GGC2* and *RGG1* were all promoted in the MIM396 plants and the GS2OE plants, in comparison with in ZH11 plants (Fig. 6A). Meanwhile, both *RGB1* and *RGG2* was up-regulated in the MIM396 plants (Fig. 6A). But *GS3* was slightly down-regulated in both the MIM396 and the GS2OE plants (Fig. 6A). Further, we checked their expression in the young embryos, as anticipated, *RGA1*, *RGB1*, *DEP1*, *GGC2*, and *RGG1* were all promoted in the MIM396 plants and the GS2OE plants, while *GS3* was obviously down-regulated in them (Fig. 6B). Thus, there is high possibility that the OsmiR396/GS2 module might also regulate the expression of all the other five grain size regulating G protein genes besides *GS3*. Considering that *RGG1* negatively regulate grain size (Tao et al. 2020), and it was promoted in the MIM396 and GS2OE plants, the regulation of OsmiR396/GS2 on this gene might not be direct. We therefore focused on the other four.

Sequence analysis of the promoters of these other four G protein encoding genes revealed that there are 17, 9, 30, and 9 putative GRF binding sites in the promoters of *RGA1*, *RGB1*, *DEP1*, and *GGC2* respectively (data not shown). To test possible transcriptional regulation of GS2 on these G protein encoding genes, we further used EMSA to check if GS2 could bind to these promoters. It was revealed that GS2 protein could indeed bind to the fragments from promoters of these four G protein encoding genes respectively (Fig. 6C), indicating possible direct regulation on these genes.

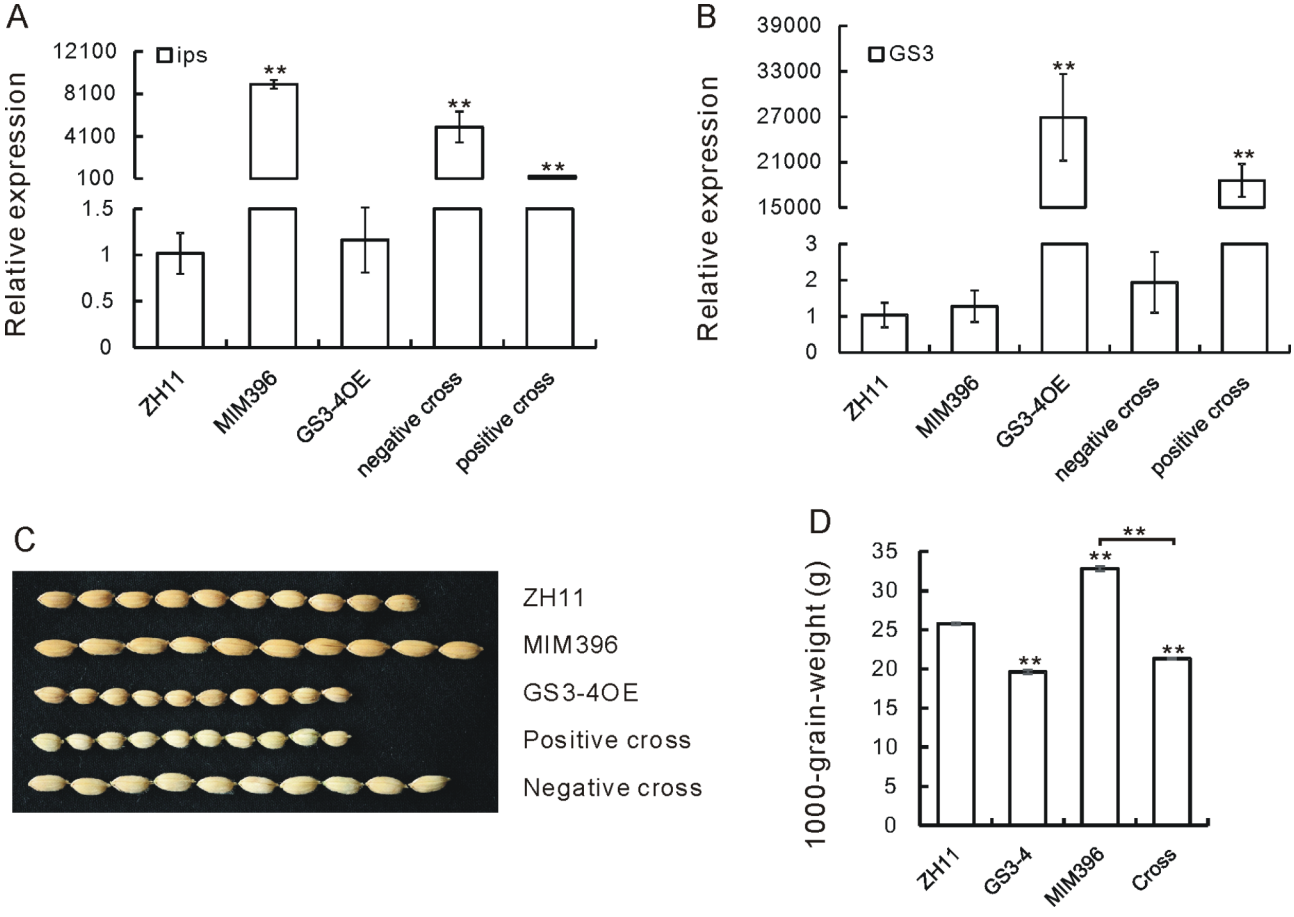

Fig. 3 Molecular detection and grain phenotype of the cross between GS3-4OE and MIM396 plants. **A**, Expression of *IPS* gene in the ZH11, GS3-4OE, MIM396, and the cross plants. Data are means ± SD ($n = 3$). **B**, Expression of *GS3* gene in the ZH11, GS3-4OE, MIM396, and the cross plants. Data are means ± SD ($n = 3$). Asterisks in (**A**) and (**B**) indicate significant differences comparing with ZH11 as determined by the Student's *t*-test (**, $P < 0.01$). **C**, Picture of 10 grains of ZH11, GS3-4OE, MIM396 and the cross plants between them. **D**, 1000-grain-weight of ZH11, GS3-4OE, MIM396 and the cross plants between them. Data are means ± SD ($n = 10$). Asterisks indicate significant differences comparing with ZH11 or as indicated as determined by the Student's *t*-test (**, $P < 0.01$)

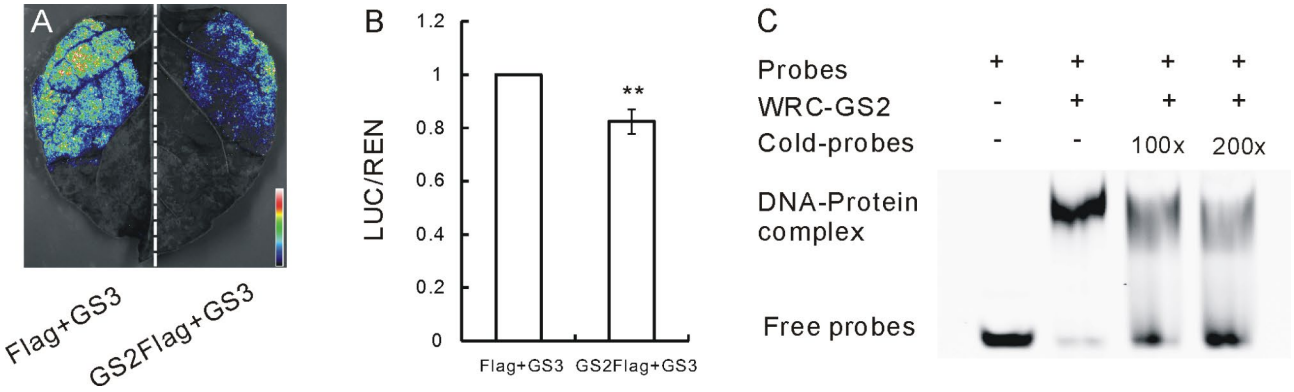

Fig. 4 Detection of transcriptional regulation of GS2 on *GS3*. **A**, Dual-LUC assay of the GS2 protein and the *GS3* promoter. **B**, The LUC/REN ratio of the Dual-LUC assay in (**A**). Data are means ± SD ($n = 3$). Asterisks indicate significant differences compared with "Flag + GS3" as determined by the Student's *t*-test (**, $P < 0.01$). **C**, EMSA of the WRC domain of GS2 binding on the promoter fragments of the *GS3* gene

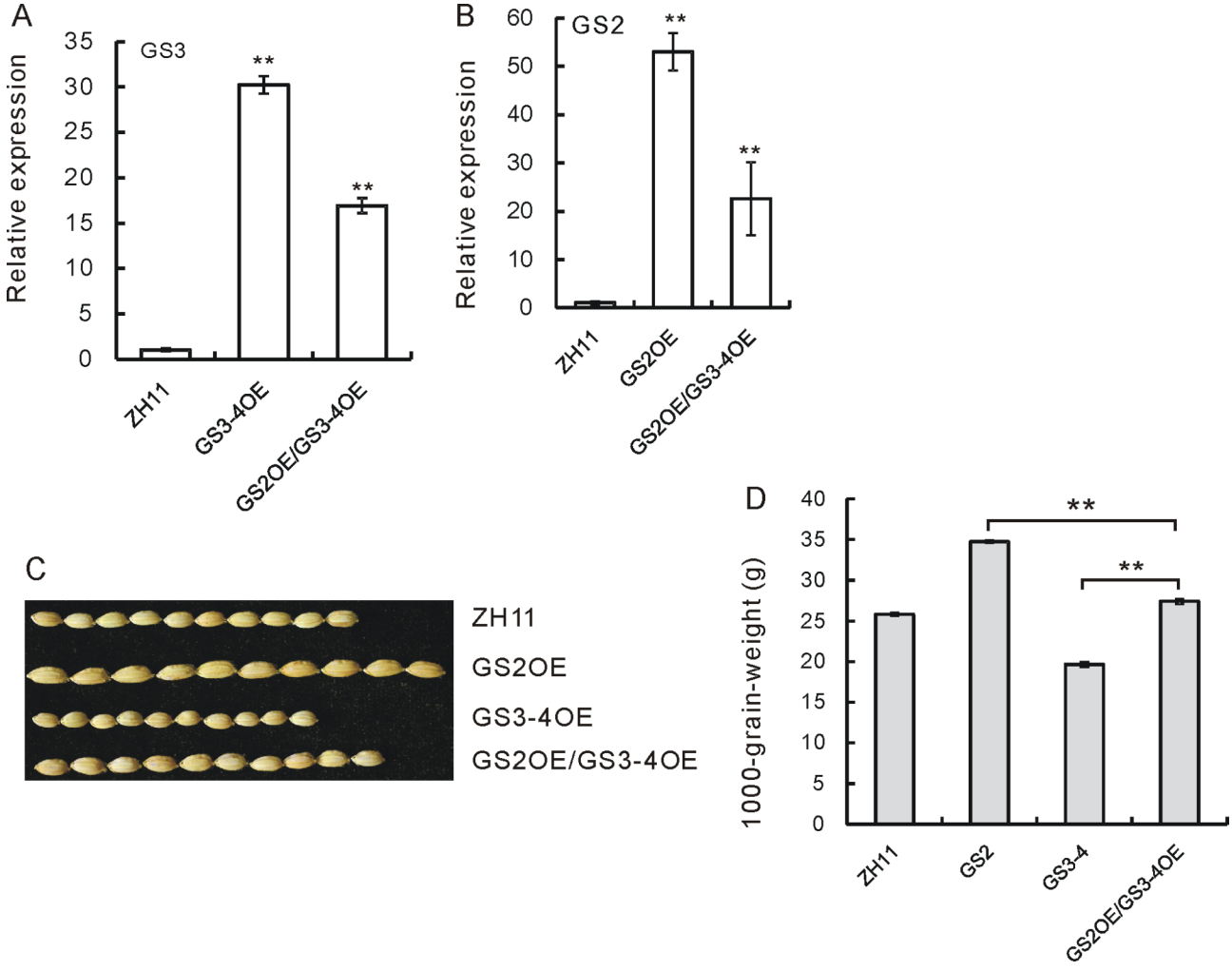

Fig. 5 Molecular detection and grain phenotype of the cross between GS3-4OE and GS2OE plants. **A**, Expression of *GS3* gene in the ZH11, GS3-4OE and GS2OE/GS3-4OE plants. Data are means ± SD ($n = 3$). **B**, Expression of *GS2* genes in the ZH11, GS2OE and GS2OE/GS3-4OE plants. Data are means ± SD ($n = 3$). Asterisks in (**A**) and (**B**) indicate significant differences with ZH11 as determined by the Student's *t*-test (**, $P < 0.01$). **C**, Picture of 10 grains of ZH11, GS2OE, GS3-4OE and the GS2OE/GS3-4OE plants. **D**, 1000-grain-weight of ZH11, GS2OE, GS3-4OE and the GS2OE/GS3-4OE plants. Data are means ± SD ($n = 10$). Asterisks indicate significant differences as indicated as determined by the Student's *t*-test (**, $P < 0.01$)

Discussion

A comprehensive exploration of the underlying molecular and genetic mechanisms of grain weight is beneficial to crop genetic improvement. Due to the significance of grain size regulation to yield promotion, great efforts have been paid to the study of molecular basis of grain size regulation and accordingly, several typical pathways have been collectively summarized (Li et al. 2019). The next question comes that if these signaling pathways could cross talk. Several members of the OsmiR156 targets have been revealed to regulate grain size, such as OsSPL14 (Jiao et al. 2010), OsSPL16 (Wang et al. 2012), OsSPL13 (Si et al. 2016) and OsSPL12 (Zhang et al. 2021). Meanwhile, several members of the targets of OsmiR396 have also been revealed to regulate grain size, such as OsGRF4 (Duan et al. 2015; Hu et al. 2015), OsGRF8 (Yang et al. 2021). And different miRNAs have been revealed to cross talk to regulate grain size. For example, OsmiR396 regulate OsmiR408 through OsGRF8 to module grain size. OsmiR159 negatively regulates grain size, and one of its pathway might through regulating OsmiR167 (Zhao et al. 2023). And the hormone signaling pathways, such as the Gibberellin (GA), the Brassinolide (BR) and auxin signaling pathways have been revealed to be extensively involved in miRNA-mediated signaling (Dai et al. 2018; Gao et al. 2018; Tang et al. 2018). Due to the transcriptional factor character of the targets of miRNAs, there is high possibility that these transcriptional factors might function in establishing a cross-talking network regulating grain size, thus many factors from other signaling pathways might be involved.

As pivotal cellular signaling elements, G proteins regulate not only growth but also many important physiological processes including abiotic stresses. For example, *GS3*

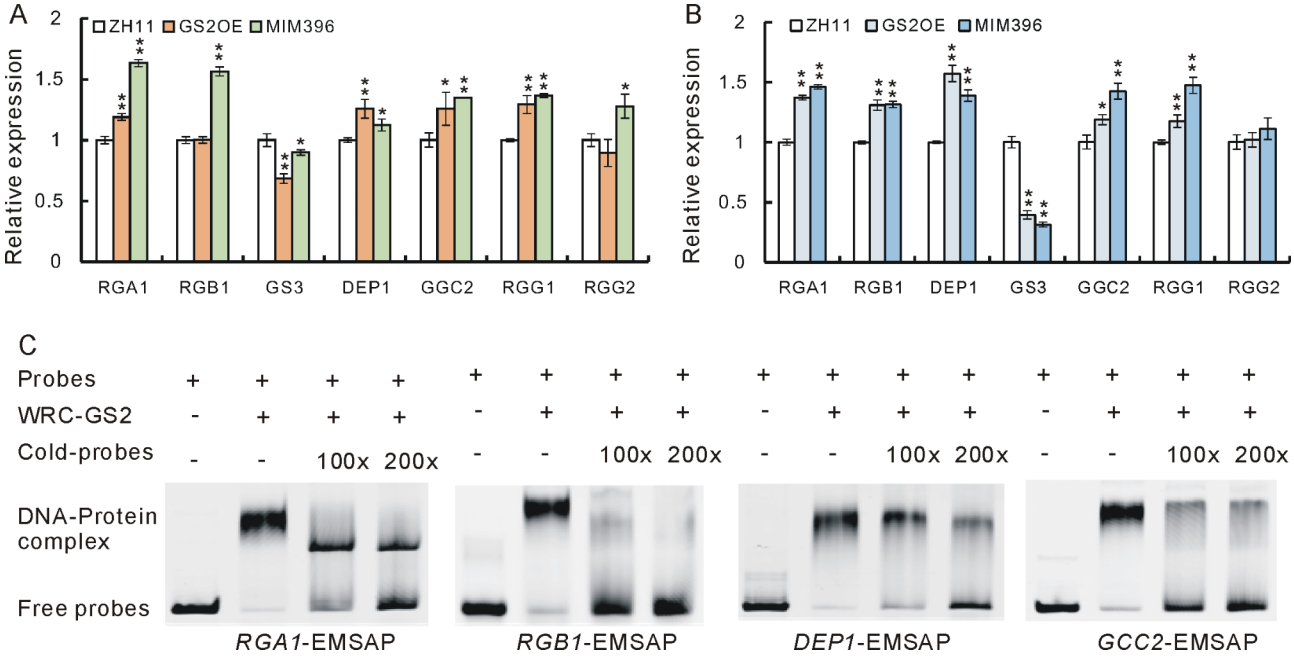

Fig. 6 Expression assays of the grain size-regulating G protein encoding genes and EMSA of the GS2 protein on the promoter fragments of these genes. **A**, qRT-PCR analysis of the *RGA1*, *RGB1*, *GS3*, *DEP1*, *GGC2*, *RGG1* and *RGG2* genes in the young panicles of the MIM396, GS2OE and ZH11 plants. Data are means ± SD ($n = 3$). **B**, qRT-PCR analysis of the *RGA1*, *RGB1*, *GS3*, *DEP1*, *GGC2*, *RGG1* and *RGG2* genes in the young embryos of the MIM396, GS2OE and ZH11 plants. Data are means ± SD ($n = 3$). Asterisks in (**A**) and (**B**) indicate significant differences with ZH11 as determined by the Student's *t*-test (**, $P < 0.01$; *, $P < 0.05$). **C**, EMSA assays of the WRC domain of the GS2 protein on the promoter fragments of *RGA1*, *RGB1*, *DEP1*, and *GGC2* genes respectively

was a typical multi-functional gene. Initial study identifies *GS3* to regulate grain size and plant architecture in rice, with over expression of the OSR domain showing obvious decrease in both grain size and plant height, while edited GS3KO plants increasing grain size. Moreover, *GS3* mediates the bridging of G-protein signaling with Ca^{2+} sensing to regulate wax metabolism, and provides a potential of improving yield under global warming (Kan et al. 2022). Furthermore, *GS3* regulates alkaline tolerance through modulating the oxidative stress caused by alkali, and this function is extensively conserved in several species such as sorghum, millet, rice, and maize (Zhang et al. 2023). It is highly possible that G proteins might function with other signaling pathways in regulating these different characters. To reveal the possible interacting signaling pathways of *GS3* in regulating grain size, we attempted to check its upstream transcriptional regulation by OsGRF4 and OsGRF8 proteins, which are the targets of OsmiR396. We revealed that *GS3* gene was obviously down-regulated in the young embryos of the MIM396 plants, the GRF8OE plants, and the GS2OE plants (Figs. 1A and B and 6B). The fact that in the young panicles of the MIM396 plants and the GS2OE plants, *GS3* was only slightly down-regulated (Fig. 6A), while in the young embryos, it was obviously down-regulated (Fig. 6B), indicated that *GS3* might be more specifically expressed and function in the young embryos to regulate grain size. Furthermore, biochemical assays revealed the possible binding of both OsGRF4 and OsGRF8 on the promoter of *GS3* gene (Fig. 1C-F, and Fig. 4). Finally, genetic relation analysis revealed that GS3-4OE could recovered the larger grain size of not only the MIM396 plants (Figs. 2 and 3), but also the GS2OE plants (Fig. 5), therefore indicating tight genetic interaction between OsmiR396/GS2 and *GS3* gene.

From the other side, as an important transcriptional factor that regulate grain size, GS2 might also regulate other genes. Accordingly, through EMSA, we revealed that GS2 might bind to the promoters of the other four G protein encoding genes that function in grain size regulation (Fig. 6C), they are *RGA1*, *RGB1*, *DEP1* and *GGC2.* This result together with the promoted expression of these four genes in the MIM396 and GS2OE plants strongly indicated that the OsmiR396/GS2 module might also regulate these G protein encoding genes genetically. Further genetic relation analysis would help give out definite conclusion. Also, *RGG1* was promoted in the GS2OE and MIM396 plants (Figs. 1A and 6B), indicating possible influence by OsmiR396. However, considering the negative role of *RGG1* in grain size regulation (Tao et al. 2020), we deduced that the relation between OsmiR396 and *RGG1* might not be direct and did not study it together with the other four G protein encoding genes.

To conclude, the OsmiR396/OsGRFs module and the *GS3* gene showed tight genetic interaction in grain size regulation. Thus, we established a bridge linking the

miRNA-transcription factors pathway and the G-protein signaling pathway that regulates grain size in rice.

Materials and Methods

Plant Species and Growth Conditions

The wild type (WT) rice plants used in this study was variety ZH11 (*Oryza sativa* L. subsp. *japonica* cv. Zhonghua No.11, ZH11). Rice plants were cultivated under field conditions at two different experimental stations in Shanghai (30°N, 121°E) and Lingshui (Hainan Province, 18°N, 110°E), China. Rice seedlings were cultures in the phytotron in CAS Center for Excellence in Molecular Plant Sciences, with 30/24±1℃ day/night temperature, 50–70% relative humidity and a light/dark period of 14 h/10 hours.

GS3-4OE and GS3KO plants were kindly gifted by Sun et al. (Sun et al. 2018).

RNA Isolation and Quantitative Real–time RT–PCR (qRT–PCR) Analysis

For gene expression analysis, such as *GS2*, *GS3* and *IPS*, seedlings were used. Total RNAs were extracted using TRIzol (Life technologies, USA) and reverse transcribed using the First Strand cDNA Synthesis Kit (Toyobo). qRT–PCR was performed with the SYBR Green Real–time PCR Master Mix Kit (Toyobo), cDNA was synthesized from 1 μg of total RNA and 1 μl of cDNA was used as template for real–time analysis. The *actin* gene was used as an internal control for normalization. Data from three biological repeats were collected, and the mean value with standard error was plotted.

All the primer sequences used in qRT–PCR and other analysis were listed in Supplementary table S1.

miRNA Northern Blot Analysis and Stem–loop qRT–PCR Analysis

miRNA Northern blot was carried out as previously described (Dai et al. 2018). Specifically, leaves of the rice seedlings were used for RNA extraction, and the OsmiR396 probes were synthesized with 5'–end Biotin. The blots were incubated at 42℃ for 30 min in the Hybridization Buffer (Ambion). And 50–80 pM probes were added in the hybridization buffer to incubate overnight. 5 S rRNA was used as RNA loading control.

Electrophoretic Mobility Shift Assay (EMSA) Assay

For protein expression and purification, the WRC domain of OsGRF4 and OsGRF8 was cloned into the pET44b vector and transformed into E. coli strain BL21 to produce His–tagged fusion protein. The His–WRC fusion protein was induced by adding 0.5 mM isopropyl–d–1–thiogalactopyranoside (IPTG) to the culture medium and incubating the cells for 14 h at 20 °C and purified using Ni–NTA (nitrilotriacetic acid) agarose (*GenScript*) according to the manufacturer's instructions. The EMSA DNA probes from *GS3* promoter were synthesized and cy5 labeled. The DNA probes and proteins were co-incubated in the reaction buffer, purified and incubated with the Cy5–labeled probe at 25 °C for 20 min in EMSA buffer (25 mM Hepes (pH 7.5), 40 mM potassium chloride, 3 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA, 0.5 mg/ml bovine serum albumin, 0.5 mg/ml poly–glutamate). After incubation, the reaction mixture was electrophoresed on a 15% native polyacrylamide gel, and then labeled DNA was detected using a Starion FLA–9000 instrument (Fujifilm, Tokyo, Japan).

Dual Luciferase (LUC) Assay

For the binding activity assays, a 1525 bp genomic fragment upstream of the *GS3* start codon ATG that contains 21 putative GRF binding motifs was cloned into the pGreenII 0800-LUC vector as the reporter. The full-length CDSs of *GS2* and *OsGRF8* were respectively cloned into pCAMBIA1300Flag-Nos and PHB vectors as effectors. The p1301Flag-Nos empty vector and PHB empty vector were used as negative controls, respectively.

All the recombinant constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (pSoup-P19). Overnight cultures were collected by centrifugation and re-suspended in MS medium to OD600=1.0, and incubated at RT for 3 h. The reporter and effectors strains were mixed at the ratio of 1:1 and infiltrated into tobacco (*Nicotiana benthamiana*) leaves and the negative control was infiltrated into the opposite position on the same leaves. Leaves were collected after 3 days (long day/white light) and infiltrated with 150 mg/mL luciferin solution; images were captured using a CCD camera 5 min later and quantification was performed using Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Three biological repeats were measured for each sample.

ChIP Assay

Immunoprecipitation of DNA associated with modified histones was carried out according to the EpiQuikTM Plant ChIP Kit (Epigentek). Rice young panicles were cross-linked in 1% formaldehyde, quenched cross-linking and washed twice in deionized water. The resulting extract was sonicated to fragment chromatin (4, 9, 10 s burst/5 min rest, 280 v) and centrifuged for 10 min at 17 500 g. Binding antibody to the assay plate and chromatin was immune-precipitated with GFP antibody. Finally, immune precipitated sample and whole-cell extract (input) were incubated at 65 °C to reverse cross-linked DNA, and ethanol precipitation to elute purified DNA. ChIP DNA and input were subjected to qRT–PCR using the primers designed to amplify a sequence in the promoter, a sequence in the coding region was used as control.

All the primer sequences used in this study were listed in Supplementary table S1.

Measurement of 1000-grain-weight

The 1000-grain-weight was got by measuring 100 grains in 10 biological repeats and converted into 1000-grain-weight. Data was shown as mean ± SD.

Primer Sequences

All the oligo sequences used in this study were listed in the Supplementary table S1.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-024-00736-6.

Supplementary Material 1

Supplementary Material 2

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Author Contributions

Zhu L, Shen Y and Dai, Z carried out the experiments, Zhu L and Shi Z analyzed the data, Miao X and Shi Z designed the experiments and wrote the MS. All authors read and approved the final manuscript.

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Data Availability

No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no competing interests.

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