OsGRF4 controls grain shape, panicle length and seed shattering in rice

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Abstract Traits such as grain shape, panicle length and seed shattering, play important roles in grain yield and harvest. In this study, the cloning and functional analysis of PANICLE TRAITS 2 (PT2), a novel gene from the Indica rice Chuchalani (CDL), is reported. PT2 is synonymous with Growth-Regulating Factor 4 (OsGRF4), which encodes a growth-regulating factor that positively regulates grain shape and panicle length and negatively regulates seed shattering. Higher expression of OsGRF4 is correlated with larger grain, longer panicle and lower seed shattering. A unique OsGRF4 mutation, which occurs at the OsmiRNA396 target site of OsGRF4, seems to be associated with high levels of OsGRF4 expression, and results in phenotypic difference. Further research showed that OsGRF4 regulated two cytokinin dehydrogenase precursor genes (CKX5 and CKX1) resulting in increased cytokinin levels, which might affect the panicle traits. High storage capacity and moderate seed shattering of OsGRF4 may be useful in high-yield breeding and mechanized harvesting of rice. Our findings provide additional insight into the molecular basis of panicle growth.

Keywords: High-yield breeding; mechanized harvesting; OsmiRNA396; panicle traits; plant growth regulator

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INTRODUCTION

Grain shape, panicle length and seed shattering, play important roles in grain yield, grain quality and harvesting. A few genes controlling grain size have been cloned over the past decades. For example, Grain Length 7 (GL7) encodes a TONNEAU1-recruiting motif protein. Upregulation of GL7 expression results in longer grain length and narrower width (Wang et al. 2015a). Grain Weight 8 (GW8) encodes a protein that is a positive regulator of cell proliferation, and enhances grain width and yield in rice (Wang et al. 2012). GW8 binds directly to the Grain Width 7 (GW7) promoter and represses its expression (Wang et al. 2015b). Grain Weight 2 (GW2) encodes a previously unknown RING-type protein with E3 ubiquitin ligase activity. The loss of GW2 function enhances grain width, weight and yield (Song et al. 2007).

Genes controlling the number of spikelets in the panicle were isolated. Grain Number 1a (GN1a) encodes cytokinin oxidase/dehydrogenase 2 (OsCKX2) that degrades the phytohormone cytokinin. Reducing the expression of OsCKX2 causes cytokinin accumulation in inflorescence meristems and increases the number of spikelets per panicle (Ashikari et al. 2005). ERECT PANICLE 3/LARGER PANICLE (EP3/LP) encodes a Kelch repeat-containing F-box protein, which modulates cytokinin levels in plant tissues, regulates the inflorescence branches and the number of spikes per panicle (Li et al. 2011b). The QTL for erect panicle on chromosome 9/DENSE AND ERECT PANICLE 1/DENSE PANICLE 1 (qPE9-1/DEP1/DN1) encodes a protein homologous to the keratin-associated protein 5-4 family, which regulates panicle, grain length and grain weight. The loss-of-function mutation of qPE9-1 leads to more erect panicles (Huang et al. 2009; Zhou et al. 2009a; Taguchi-Shiobara et al. 2011). In fact, many panicle trait genes are pleiotropic. For example, Ghd7.1 (Yan et al. 2013) and Ghd8 (Yan et al. 2011) control the number of spikelets, affecting the plant height and heading date.

Several genes determining seed shattering were characterized. Shattering1/Shattering4 (SHAI/SH4) encodes a member of the trihelix family of plant-specific transcription factors. Wild rice disperses seeds freely at maturity, unlike the domesticated rice cultivars that have lost the ability to shed their seeds at maturity because of a single amino acid substitution (Lin et al. 2007). The qSH1 (QTL of seed shattering in chromosome 1) encodes a BEL1-type homeobox gene. It represents a single-nucleotide polymorphism (SNP) causing loss of seed shattering owing to the absence of abscission.
layer formation (Konishi et al. 2006; Zhang et al. 2009). SHATTERING ABORTION1 (SHAT1) encodes an APETALA2 transcription factor, which is required for seed shattering via abscission zone (AZ) development in rice, positively regulated by the trihelix transcription factor SH4. The qSH1 acts downstream of SHAT1 and SH4 (Zhou et al. 2012). Shattering5 (SH5) is highly homologous to qSH1, which induces the expression of SHAT1 and SH4 (Yoon et al. 2014). During mechanical harvest, high seed shattering leads to increased loss of production.

Although the genes affecting panicle traits were cloned, the molecular basis of panicle growth is still unclear. In this study, we cloned and functionally analyzed OsGRF4, an allele that shows extraordinary effect on rice panicle traits. Evidence suggests that OsGRF4 is downregulated by miR396 during grain development. OsGRF4 from Indica rice Chuandali (CDL) carries a mutation in the coding sequence targeted by OsmiRNA396, which enhances the expression levels of OsGRF4, and results in increased grain length, grain width, grain weight, panicle length and reduced seed shattering. Based on our findings, OsGRF4 can be used in breeding new varieties to improve rice grain yield and the seed shattering.

RESULTS

Map-based cloning of PT2

We constructed a recombinant inbred line (RIL) and a nearly isogenic line (NIL) population from a cross between cultivar R1126 (medium-grain) and CDL (big-grain). High-resolution mapping with homozygous recombinant plants was carried out. The PT2-containing region was delimited to approximately 33.2 kb between the markers GL2-35-1 and GL2-12 in the long arm of rice chromosome 2 (Zhang et al. 2013). In this candidate genomic region, there were three predicted gene loci (LOC_Os02g47280, LOC_Os02g47290 and LOC_Os02g47300) according to the Rice Annotation Project (Kawahara et al. 2013). LOC_Os02g47280 encodes a putative growth-regulating factor OsGRF4, which belongs to the GRF protein family. The GRF protein family contains two conservative domains: WRC (Trp, Arg, Cys) and QLQ (Gln, Leu, Gln), which mediate DNA binding and protein interaction, respectively (Kim et al. 2003). Previous studies have shown that the members of this family regulate rice growth, heading stage, seed development and resistance (Esther et al. 2000; Ye et al. 2004; Luo et al. 2005; Gao et al. 2010), as well as growth of cotyledons, leaves and pistil in Arabidopsis (Kim et al. 2003). LOC_Os02g47290 and LOC_Os02g47300 encode a putative uncharacterized protein, without any gene ontology. Therefore, OsGRF4 is the most probable candidate gene to PT2.

Confirmation of OsGRF4 as PT2

To determine whether OsGRF4 represented the PT2, we generated transgenic plants expressing OsGRF4 (CDL) in the NIL-PT2. We introduced the plasmid carrying OsGRF4 (CDL) (designated gPT2), which contained a 4.07-kb genomic DNA fragment into NIL-PT2, driven by the ubiquitin promoter. A total of 28 putative transgenic plants (T0) were generated, and their genotypes were determined by polymerase chain reaction (PCR) amplification of the hygromycin phospho-transferase gene, of which 18 were positive transgenic lines, whereas the other 10 were negative. We observed an increase in grain size, 1000-grain weight, panicle length and changes in seed shattering of the positive transgenic plants (T1), compared with the negative plants, without affecting the plant morphology. In contrast, the OsGRF4 RNA interference (RNAi) transgenic plants in the NIL-PT2 background generated medium grains with easy seed shattering (Figures 1, 2). A comparison of relative expression of the OsGRF4 gene overexpression and RNAi transgenic plants (T1) showed that plants with higher OsGRF4 expression levels produced bigger

![Figure 1. Comparison of panicle traits in transgenic plants](image)

(A) Grains of OX (+) and OX (−), Scale bar = 10 mm. (B) Grains of RNAi (+) and RNAi (−), Scale bar = 10 mm. (C) Panicles of OX (+) and OX (−), Scale bar = 10 cm. (D) Panicles of RNAi (+) and RNAi (−), Scale bar = 10 cm. OX, overexpression; RNAi, RNA interference; (+) indicates positive transgenic T1 plants; (−) indicates negative transgenic T1 plants.
and heavier grains (Figure 2). Therefore, OsGRF4 represents the gene PT2.

**Sequence differences in OsGRF4**

The full-length complementary DNA (cDNA) of OsGRF4 was isolated by reverse transcription-PCR (RT-PCR) from R1126 and CDL. Alignment of the cDNA sequence with the genomic sequence of Nipponbare indicated that OsGRF4 consists of six exons in R1126 and five exons in CDL (Figure 3). We compared the genomic sequences corresponding to the open reading frame (ORF) and the promoter regions of OsGRF4 between R1126 and CDL, and found that the coding sequence of CDL was 1,185 bp in length, encoding a predicted polypeptide of 394 amino acids, whereas the coding sequence of R1126 was 1,140 bp, encoding a polypeptide of 379 amino acids (Figure S1). There were six nucleotide differences in the coding sequences between R1126 and CDL, resulting in the substitution of three amino acids (Figure 3). The OsGRF4 gene contains a MicroRNA396 target sequence in the coding region, with two variable bases (AA - TC) between the target
sequence of R1126 and CDL. The GRF4 gene is repressed by elevated levels of MicroRNA396 (Jones-Rhoades and Bartel 2004). A comparison of the promoter sequences revealed 20 polymorphisms in the 2-kb region upstream of the translation starting site, including substitutions, deletions and insertions (Figures 3, S2).

Expression of OsGRF4 in NILs

We compared the expression profiles of OsGRF4 in various organs of NILs by quantitative RT-PCR analysis with total RNA. The OsGRF4 transcript levels varied drastically among the tissues. OsGRF4 was preferentially expressed in developing panicles, and the highest levels of expression were found in panicles of 7 cm in length. On the other hand, there was less transcript accumulation in the rice hull, root, stem and leaf sheath. In particular, the transcript was much more abundant in NIL-OsGRF4 than in NIL-Osgrf4 in the young panicles measuring 1 cm, 4 cm, 7 cm, 11 cm and 15 cm in length (Figure 4A). The differences corresponded with the critical stages of panicle traits. The OsGRF4 effect on panicle traits might be attributed to differences in expression levels.

Sequence polymorphisms of OsGRF4 in rice germplasm

Twenty-five rice germplasms with abundant diversity in grain shape were selected for sequencing promoter regions and coding sequences that cover the four mutation sites in the exon of OsGRF4, and measured the grain length in a total of 25 rice accessions (Tables 1, S1). The coding sequence of the cultivated varieties was divided into two basic haplotypes: Indica-type and Japonica-type. The OsGRF4 of most Indica varieties belongs to Indica-type and the OsGRF4 of most Japonica varieties represents the Japonica-type. Some varieties, such as R1126 and R299, are Indica varieties but their OsGRF4 belongs to Japonica-type. These varieties are usually produced from interspecies crosses between Indica and Japonica. The OsGRF4 of CDL belongs to Indica-type, but an unusual mutation (AA-TC) occurred between the CDL and Indica-type. Similar types as OsGRF4 were not found within the 25 rice accessions and the database (http://ricemvarmap.ncpgr.cn) involved over a thousand varieties. Therefore, the OsGRF4 of CDL was unique. The distinct mutation at the OsmiRNA396 target site of OsGRF4 may enhance the expression of OsGRF4, and result in improved grain shape, panicle length and seed shattering.

The promoter sequence of the cultivated varieties represents a wide diversity, and comprises six haplotypes: CDL, Gang 46B, R700, Fengyuan B, R1126 and R299 types. Six varieties (Yuzhenxiang, Yuzhuxiang, Nongxiang 18, Xiangwanxiang7, Nongxiang 21 and Nongxiang 29) of CDL type represent good quality rice with long and narrow grain. OsGRF4 may not be an important gene contributing to the long grain of the six varieties.

To determine the specific mutation or promoter regions of OsGRF4 responsible for the phenotypic variation, we expressed the cDNA of OsGRF4 from Nongxiang 18 (Indica-type) and R1126 (Japonica-type) respectively, driven by the promoter of CDL. A total of 39 transgenic plants were obtained from pOsGRF4CDL::OsGRF4JcDNA, of which 22 were transgene positive, whereas the other 17 were negative. Forty-one transgenic plants were obtained from pOsGRF4CDL::OsGRF4Indica, of which 36 were transgene positive, whereas the other five were negative. We observed no increase in grain length in the transgene-positive plants, compared with the NIL-Osgrf4 (Table S2). This result showed that the polymorphisms in the OsGRF4 promoter region may not be responsible for the phenotypic variation. Collectively, the results from the sequencing, expression pattern and transformation studies suggest that the specific mutations in the OsGRF4 coding sequence may determine the grain shape, panicle length and seed shattering.

Biological roles and characterization of OsGRF4

We also examined the inner and outer surfaces of glumes between NILs using a scanning electron microscope (Figure 4B–E). The cell length and width of rice inner surface in NIL-OsGRF4 was much shorter than in NIL-Osgrf4 (Figure 4F). In addition, the number of rice inner surface in NIL-OsGRF4 was also higher than in NIL-Osgrf4, although differences were not significant (Figure 4C). Therefore, it is very likely that OsGRF4 positively regulated grain size mainly by increasing cell length partially and cell number, leading to enhanced longitudinal growth in the grain.

The anatomical structure of the abscission zone in rice pedicels was investigated by optical microscopy (Figure S3). NIL-OsGRF4 had a partially developed abscission zone, but NIL-Osgrf4 showed a well-developed abscission zone, indicating that OsGRF4 gene improved seed shattering via differential abscission zone formation.
Large spikelet hulls are associated with incomplete grain filling (Song et al. 2007). Therefore, the NILs for grain filling rate were compared by measuring the fresh and dry weight of the grains during grain filling (Figure S4). Both fresh and dry weights of NIL-OsGRF4 were significantly higher ($P < 0.01$) than those of NIL-Osgrf4 at day 1 after fertilization, and the differences peaked at approximately 10 d after fertilization, when dry weights of the grains of NIL-OsGRF4 were 59.69% higher than that of NIL-Osgrf4. Thus, the increase in grain weight and yield per plant

Figure 4. Comparison of OsGRF4 expression pattern and glume traits in NILs
(A) RH2, RH4 and RH7 represent rice hulls at reproductive stage in 2-cm, 4-cm and 7-cm spikelet; 1YP, 4YP, 7YP, 11YP and 15YP are young panicles measuring 1 cm, 4 cm, 7 cm, 11 cm and 15 cm length; R, S, FL and LS represent root, stem, flag leaf and leaf sheath at vegetative stage (VS) and reproductive stage (RS). All data are based on experiments in duplicate and expressed as means ± SD. Scanning electron microscopy analysis of glume inner (B, C) and outer (D, E) surfaces of NILs spikelets at mature stage. Scale bars = 100 μM. Cell length (F) and cell number (G) of glume inner surfaces of NILs spikelets at mature stage.
resulted from increase in both grain shape and grain filling rate.

To determine the sub-cellular localization of OsGRF4, the coding sequence of OsGRF4 was fused with yellow fluorescent protein (YFP). In contrast, Ghd7, which is a nuclear protein, was fused with cyan fluorescent protein (CFP). Both fluorescent proteins were individually driven by the constitutive 35S cauliflower mosaic virus promoter. The constructs were co-transfected into rice protoplasts of etiolated seedlings by polyethylene glycol. The result showed that OsGRF4 co-localized to the nucleus with Ghd7 (Figure 5).

OsGRF4 genetically regulates cytokinins, cell cycle and panicle traits

We analyzed the expression of 25 genes involved in cell cycle (14 putatively involved in the G1/S and 11 in the G2/M phase), 13 panicle trait-related genes and three genes in the cytokinin biosynthesis. The transcription levels of six cell cycle genes (MAD2, MCM4, CYCB2.1, CYClα2m, CDKB, and KN) and two panicle trait genes, Grain Size 5 (G5) and Gmta, were greatly elevated in NIL-OsGRF4 when compared with NIL-Osgrf4 plants (Figure 6A–C). In contrast, the expression of two cell cycle genes (CAK1 and CYCT1) and 2 cytokinin-related genes (CKX5 and CKX1) was significantly reduced in the NIL-OsGRF4 plants, relative to NIL-Osgrf4 plants (Figure 6A, D).

Hormones play important roles in the formation of panicle architecture, especially cytokinin. Cytokinin regulates cell growth and development. The cytokinin content regulates rice grain production (Ashikari et al. 2005). The possible

| Accessions   | Class     | Grain length (mm) | SNP1 | SNP2 | SNP3 | SNP4 | Promoter haplotype   |
|--------------|-----------|-------------------|------|------|------|------|----------------------|
| CDL          | Indica    | 13.52             | A    | T    | T    | A    | CDL type             |
| NIL-OsGRF4   | Indica    | 12.67             | A    | T    | A    | T    | CDL type             |
| Nongxiang 99 | Indica    | 11.52             | A    | T    | T    | T    | CDL type             |
| Yuzhenxiang  | Indica    | 13.28             | A    | T    | T    | T    | CDL type             |
| Yuzhuxiang   | Indica    | 12.74             | A    | T    | T    | T    | CDL type             |
| Nongxiang 18 | Indica    | 11.73             | A    | T    | T    | T    | CDL type             |
| Xiangwan xian17 | Indica | 11.60             | A    | T    | T    | T    | CDL type             |
| Nongxiang 21 | Indica    | 11.74             | A    | T    | T    | T    | CDL type             |
| Nongxiang 29 | Indica    | 12.46             | A    | T    | T    | T    | CDL type             |
| Gang 46B     | Indica    | 7.81              | A    | T    | T    | T    | Gang 46B type        |
| Nongxiang 16 | Indica    | 9.37              | A    | T    | T    | T    | Gang 46B type        |
| BL122        | Indica    | 8.41              | A    | T    | T    | T    | Gang 46B type        |
| Jiafuzhan    | Indica    | 11.46             | A    | T    | T    | T    | Gang 46B type        |
| Minghui 86   | Indica    | 10.00             | A    | T    | T    | T    | Gang 46B type        |
| Xinyinzhan   | Indica    | 8.06              | A    | T    | T    | T    | Gang 46B type        |
| R700         | Indica    | 9.31              | A    | T    | T    | T    | R700 type            |
| Fengyuian B  | Indica    | 9.95              | A    | T    | T    | T    | Fengyuian B type     |
| R1126        | Indica    | 10.91             | G    | G    | C    | G    | R1126 type           |
| NIL-Osgrf4   | Indica    | 10.49             | G    | G    | T    | G    | R1126 type           |
| Nanyangshan  | Indica    | 12.42             | G    | G    | C    | G    | R1126 type           |
| R299         | Indica    | 9.92              | G    | G    | T    | G    | R299 type            |
| 02428        | Japonica  | 7.59              | G    | G    | T    | G    | R299 type            |
| C418         | Japonica  | 9.18              | G    | G    | T    | G    | R299 type            |
| P7144        | Japonica  | 9.56              | G    | G    | T    | G    | R299 type            |
| CY016        | Japonica  | 8.64              | G    | G    | T    | G    | R299 type            |
The contribution of cytokinins to the bigger grains in NIL-OsGRF4 was determined by monitoring their levels using 7 cm young panicles of NILs. We found that the four kinds of cytokinins (isopentenyladenine riboside, trans-zeatin-riboside, cis-zeatin and cis-zeatin-riboside) showed significant differences between the two OsGRF4 alleles (Table 2). The high levels of cytokinin regulated by cytokinin-related genes might result in larger grain, longer panicle and lower seed shattering.

**OsGRF4 enhances rice storage capacity and improves seed shattering**

To test whether OsGRF4 affects grain yield, we investigated the effects of OsGRF4 on grain shape, 1000-grain weight, panicle length and seed shattering. Compared with NIL-Osgrf4, the grains of NIL-OsGRF4 were 12.23% wider and 34.32% heavier leading to a 30.37% increase in storage capacity per plant. It was also discovered that the panicle length, primary branch number and seed setting percentage showed significant differences between NILs. No significant differences were detected in other agronomic traits (Table 3), and the plant type of NIL-Osgrf4 was similar to NIL-OsGRF4 (Figure 7A–C). Interestingly, seeds of NIL-Osgrf4 were easier to thresh than those of NIL-OsGRF4. To test the shattering degree of NIL seeds, we measured the breaking tensile strength (BTS), which was inversely proportional to shattering degree. The Student’s t-test showed significant differences.

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**Figure 6. Effect of OsGRF4 on genes expressed in cell cycle, panicle traits and cytokinins**

(A) Cell cycle genes. (B, C) Panicle trait genes. (D) Cytokinins genes. The analysis of relative expression ratios was determined by qRT-PCR using 7-cm young panicles. All data are expressed as means ± SD based on experiments in triplicate. * indicated significantly different (Student’s t-test, P < 0.05).
Table 2. Comparison of hormone levels in NIL-OsGRF4 and NIL-Osgrf4

| Hormone                        | NIL-Osgrf4 | NIL-OsGRF4 |
|--------------------------------|------------|------------|
| N6-isopentenyladenine (IP)     | 0.062 ± 0.008 | 0.074 ± 0.007 |
| Isopentenyladeninriboside (IPR)| 0.402 ± 0.036 | 0.775 ± 0.033** |
| trans-zeatin (tZ)              | 0.165 ± 0.019 | 0.174 ± 0.021 |
| cis-zeatin (cZ)                | 0.056 ± 0.007 | 0.106 ± 0.014** |
| trans-zeatin-riboside (tZR)    | 0.299 ± 0.012 | 0.339 ± 0.025* |
| cis-zeatin-riboside (cZR)      | 0.334 ± 0.014 | 0.488 ± 0.067** |
| indole acetic acid (IAA)       | 47.38 ± 5.83  | 50.59 ± 2.05  |
| abscisic acid (ABA)            | 13.28 ± 0.74  | 14.61 ± 1.69  |

The content of eight hormones was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using 7-cm-long young panicles from at least 12 plants, in at least triplicate. All data were expressed as means ± SD. * and ** indicated that phenotypes between NILs were significantly different (Student’s t-test, P < 0.05 and 0.001, respectively).

Table 3. Grain shape and yield component traits of the two nearly isogenic lines (NILs)

| Traits                                | NIL-Osgrf4     | NIL-OsGRF4     |
|---------------------------------------|----------------|----------------|
| Panicle length (cm)                   | 21.42 ± 0.51   | 25.54 ± 0.74***|
| Primary branch number                 | 10.4 ± 0.55    | 11.4 ± 0.89    |
| Secondary branch number               | 19.8 ± 2.39    | 18.0 ± 2.45    |
| Spikelet number per panicle           | 125.4 ± 6.73   | 126.2 ± 10.31  |
| Grain number per panicle              | 119.2 ± 8.23   | 113.0 ± 10.77  |
| Seed setting percentage               | 95.0 ± 1.88    | 89.47 ± 1.83***|
| Tiller number                         | 6.26 ± 1.43    | 5.91 ± 1.87    |
| Grain length (mm)                     | 10.49 ± 0.26   | 12.67 ± 0.20***|
| Grain width (mm)                      | 2.78 ± 0.13    | 3.12 ± 0.04***|
| 1,000-grain weight (g)                | 31.7 ± 0.34    | 42.58 ± 1.58***|
| Storage capacity per plant (g)        | 27.82 ± 1.39   | 36.27 ± 3.25***|
| Plant height (cm)                     | 108.68 ± 1.2   | 109.03 ± 1.77  |

All data were derived from the two NILs planted in random block design in triplicate. All data were expressed as means ± SD (n = 5 plants). * and *** indicated that phenotypes between NILs were significantly different (Student’s t-test, P < 0.05 and 0.001, respectively).

(P < 0.05) in the pulling strength between NILs in three different periods (Figure 7D–F). These results showed that the shattering degree in NIL-Osgrf4 was significantly harder than in NIL-OsGRF4. Since the storage capacity was the basis of yield, and medium seed shattering reduced the loss during mechanized harvesting, the Osgrf4 was very useful in breeding high-yield rice and mechanized harvesting.

DISCUSSION

Panicle traits controlled by quantitative trait loci (QTLs) are complex yield determinants in rice. The molecular mechanisms underlying panicle traits are still unclear. Therefore, identification of QTLs that regulate panicle traits and characterization of the underlying genes enhance our understanding of rice panicle development. In this study, we reported OsGRF4 as a novel gene controlling grain shape, panicle length and rice seed shattering. The functional characterization of OsGRF4 provides a novel insight into the mechanisms controlling panicle traits. We identified the sequence differences in both the promoter region and coding sequence between R126 and CDL. Expression of OsGRF4<sup>cdna</sup> or OsGRF4<sup>cDNA</sup> driven by the CDL promoter revealed no changes in grain length, which indicates that the coding sequence targeted by OsmiRNA396 in CDL may be responsible for phenotypic variation. It was shown that OsGRF4 is downregulated by miR396 during grain development in rice (Lan et al. 2012). Specific OsGRF4 mutations may resist the regulation by OsmiRNA396, leading to enhanced OsGRF4 expression levels, and resulting in increased grain shape, panicle length and reduced seed shattering. It was reported that OsGRF4 control grain size by activating brassinosteroid responses (Che et al. 2015), and a rare mutation in OsGRF4 affecting the binding site of OsmiR396 results in large grains (Duan et al. 2015; Hu et al. 2015). A point mutation in OsSPL14 disrupts OsmiR166-directed regulation of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14 (OsSPL14), and higher expression of OsSPL14 in the reproductive stage promotes panicle branching and higher grain yield in rice (Jiao et al. 2010; Miura et al. 2010). The regulation of OsGRF4 by microRNA may be similar to OsSPL14, which requires further investigation.

There are three types of molecular mechanisms affecting phenotypic variation in the cloned genes that control grain size. The first mechanism is negative regulation of grain size. An early stop codon from a substitution in the exon of Grain Size 3 (GS3) results in large grains. GS3 acts as a negative regulator of grain size (Fan et al. 2006). Deletion of 1-bp in THOUSAND-GRAIN WEIGHT 6 (TGW6) exon results in a premature stop codon, and the functional loss of TGW6 increases grain weight and yield (Ishimaru et al. 2013) as well as grain enlargement, which is true for GW2, QTL for seed width on chromosome 5/Grain Weight 5 (qGW5/GW5) and QTL for Grain Length 3/Grain Length 3.1 (qGL3/GL3.1) (Song et al. 2007; Shomura et al. 2008; Weng et al. 2008; Zhang et al. 2012). Promoter region variation is another kind of natural mutation. It is reported that GS5 regulates the grain size via polymorphisms in the promoter region, and higher expression of GS5 results in larger grains, suggesting that GS5 positively regulates grain shape (Li et al. 2011a). Similarly, GW8 affects grain size due to a critical polymorphism in the promoter region (Wang et al. 2012). As a positive regulator of the traits, increased Grain Weight 6a (GW6a) expression enhances grain weight and yield (Song et al. 2015). Copy number variants (CNVs) contribute to phenotypic variation of various traits. A CNV on Grain Length on Chromosome 7 (GL7) locus contributes to diversity in grain size in rice (Wang et al. 2015a). Our findings provide a new molecular mechanism controlling grain shape, panicle length and seed shattering. We also found that GS5 and Gnra were greatly elevated in NIL-OsGRF4 plants when compared with NIL-Osgrf4. Studies investigating OsGRF4 regulation of the specific genes will facilitate our understanding of the regulatory network of genes encoding panicle traits.

The unusual mutation of OsGRF4 in CDL was not found in more than 20 rice cultivars sequenced in this study and over 1,000 varieties in the database, which indicates that the allele...
OsGRF4 in CDL is a new untapped genetic resource. As a pleiotropic gene, it not only significantly increases rice storage capacity, but also decreases seed shattering. Such agronomic traits are strongly desirable in rice breeding. However, the substantial increase in grain width leads to deterioration in the quality of rice morphologically, while increasing grain length reduces head rice rate. Therefore, we propose three ways to utilize OsGRF4 gene: Breeding specific high-yielding rice cultivars to produce rice flour, beer and so on; gene polymerization to reduce grain width and cultivate high-yield and high-quality rice with extra-long grain; polymerizing small grains, narrow grains or special genes to breed sterile lines with small grains, and crossbreeding the sterile lines with long and thin grain restorer lines containing large grain gene OsGRF4 to obtain high yield and good quality rice with thousand-grain weight in 30–35 g. Mechanization of rice hybrid seed production based on grain length differences between sterile lines and restorer lines can also be accomplished concurrently.

**MATERIALS AND METHODS**

**Field planting and grain shape measurement**
Harvested rice (*Oryza sativa* L.) grains were air-dried and stored at room temperature for at least 3 months before testing. Fully-filled grains were used for measuring grain width, length and weight. Ten randomly chosen grains from each plant were assembled along a vernier caliper to measure grain width and length. Grain weight was calculated based on 200 grains and converted to 1,000-grain weight.

**Characterization of shattering degree phenotype**
In order to evaluate the shattering degree of NIL-OsGRF4 and NIL-Osgrf4 phenotypes, three panicles from the main stem of each plant were harvested at 20, 30 and 40 d after full heading, respectively. The breaking tensile strength (BTS) upon detachment of grain from the pedicels by hand pulling was measured using a digital force gauge (*Qin et al. 2010*). In an individual plant, 20 grains on the uppermost part of each panicle were measured.
Vector construction and transformation

The full-length genomic DNA of OsGRF4 was isolated by PCR with primer 1390DL-1 from CDL, and then subcloned into the pCUbi1390 binary vector. The gene fragment was driven by ubiquitin promoter and the resultant plasmid was introduced into NIL-pt2 by means of Agrobacterium tumefaciens-mediated transformation (Hiei et al. 1994). The genotype of transgenic plants was determined by PCR amplification of the hygromycin phosphotransferase gene (hpt) and the analysis of hygromycin resistance.

For OsGRF4-RNAi constructs, a 290 bp fragment was isolated by PCR from vector pNW55 with the following primers: I miR-s (agtaaaacgttgacatcctcctcgagagtagctgtaag), II miR-a (tgaagggagtctaaagtttcctgtctgctacagcc), III miR’s (ctaaaagatctcaacctctttgtctgtcctagctg) and IV miR*a (aatatatggtgatctcaacggagcagggccttgaag). This fragment was cloned into the plant RNAi vector pCUbi1390. The resultant plasmid was introduced into NIL-PT2.

The chimeric construct (pOsGRF4CDL::OsGRF4IcDNA and pOsGRF4CDL::OsGRF4JcDNA) was prepared in which the 2-kb promoter fragment of OsGRF4 from CDL was fused with the cDNA from Nongxiang 18, containing a coding sequence belonging to the Indica-type (IcDNA), and R1126 whose coding sequence belonged to the Japonica-type (JcDNA), respectively. The promoter fragment was ligated with the cDNA from Nongxiang 18 and R1126, respectively, and then inserted into the pCUbi1390 binary vector. The constructs were transferred into NIL-Osgrf4 by Agrobacterium tumefaciens-mediated transformation (Hiei et al. 1994).

Expression analysis

Total RNA was extracted from various rice tissues using TRIzol reagent (Invitrogen) and was reverse transcribed using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix according to the manufacturer’s instructions. RT-PCR was performed according to Jiang et al. (2007). All assays were repeated at least three times, and Ubiquitin 5 (UBQ5) was used as a reference. The relative expression was analyzed according to Schmittgen et al. (2008). Relevant PCR primers sequences are listed in Tables S3, S4. The qPCR primers involved in cell cycle were selected from the previously reported work (Li et al. 2011a).

Histological observation

Observation of the rice glume traits: The spikelets of NIL-OsGRF4 and NIL-Osgrf4 at mature stage were collected and treated with 2.5% (vol/vol) glutaraldehyde solution, vacuumed three times, and fixed for 24 h as described by Ray (1988). The inner and outer surfaces of glumes of the spikelets were observed with a scanning electron microscope S-3000N at an accelerating voltage of 7 kV. Observation of the rice abscission zone: Panicles were harvested about 30–40 d after heading. The abscission zone of the pedicel was investigated as described by Ji et al. (2006) using an optical microscope after staining with Fast Green FCF and Safranine.

Sub-cellular localization of OsGRF4

The coding sequence of OsGRF4 (CDL) was fused with PM999–YFP. The fusion protein with the insertion in the right direction was co-transfected into rice protoplasts with Ghd7:CFP as described by Zhou et al. (2009) with minor modifications. The fluorescent image was obtained using a confocal microscope (Leica, Germany) after incubating the transformed cells in the dark at 28°C for 20 h.

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AUTHOR CONTRIBUTIONS

H. D. and L. Y. designed the study. P. S., W. Z., Y. W., Q. H., F. S., H. L., J. W. and J. W. performed the experiments. P. S. and W. Z. analyzed the data and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online
version of this article at the publisher’s web-site.

Figure S1. The predicted protein sequences of the two OsGRF4
alleles

Variant amino acids between the two parents are in green and
red color.

Figure S2. DNA sequences of the 2-kb promoter region of
OsGRF4 between the two parents R1126 and CDL

Variant SNPs and InDels between the two types are in red or
green color.

Figure S3. Longitudinal sections of abscission zone in grain
pedicel tissues

(A, B) NIL-OsGRF4. (C, D) NIL-Osgrf4. (A, C) ×100 magnifica-
tion. (B, D) ×400 magnification. AZ, abscission zone; F, flower
side; P, pedicel side.

Figure S4. Time-course of grain weight between NIL-OsGRF4
and NIL-Osgrf4 plants (n = 60 grains for each point)

Table S1. Genomic polymorphisms of OsGRF4 promoter area in
25 accessions

Table S2. Grain length (mm) of transgenic plants and NIL-
Osgrf4

Table S3. Primers used for sequencing, cloning and qPCR of
OsGRF4

Table S4. Primer sets used for qRT-PCR of the 16 genes
involved in panicle traits and cytokinins