# Transcriptome analysis of near-isogenic line provides novel insights into genes associated with panicle traits regulation in rice

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## Abstract

Panicle traits in rice impact yield and quality. The *OsGRF4* gene encodes a growth-regulating factor controlling panicle traits, and was recently cloned. Gene expression profiling analysis can be used to study the molecular mechanisms underlying *OsGRF4* regulation. Use of near-isogenic lines (NILs) reduces genetic background noise in omics studies. We compared transcriptome profiling of 7 cm long young panicles of NIL-*Osgrf4* and NIL-*OsGRF4* using RNAs sequence analyses. Eighty differentially expressed genes (DEGs) were identified. Our target gene *OsGRF4* was up-regulated in NIL-*OsGRF4* plants, which is consistent with a previous qPCR analysis. Hierarchical cluster analysis showed *OsGRF4* is tightly clustered with the up-regulated DEG LOC_Os02g47320. Gene Ontology (GO) and KEGG analysis suggested that DEGs were primarily involved in somatic embryogenesis and chitinase activity. Two up-regulated DEGs, LOC_Os04g41680 and LOC_Os04g41620, were significantly enriched in the top 8 GO terms, and were over_represented in term of seed development, and may play key roles in grain shape regulation. The transcription factor *Osmyb1* also exhibited differential expression between NILs, and may be is an important regulator of panicle traits. By searching reported functions of DEGs and by co-localization with previous identified quantitative trait loci (QTL), we determined that the pleiotropic gene *OsGRF4* may also be involve in abiotic stress resistance. This study provides new candidates genes for further understanding the molecular mechanisms underlying rice panicle trait regulation.

## Introduction

Rice is a crucial agronomic crop worldwide, especially in Asians. By the year 2030, a 40% increase in rice production will be necessary to accommodate the rapidly increasing world population [1]. Panicle traits such as grain size, panicle shape, seed shattering, and seed germination, are the main determinants of yields and quality in rice. Recent developments in molecular genetics approaches have led to cloning of many panicle trait genes in rice. Plant hormones such as cytokinin, auxin, and brassinosteroid (BR) play an importance role in rice

panicle development. *Grain number 1a* (*Gn1a*) was the first cloned major QTL responsible for grain number in rice. *Gn1a* encodes a cytokinin oxidase/dehydrogenase (*OsCKX2*), which can reduce the content of phytohormone cytokinin. Low expression level of *Gn1a* causes cytokinin accumulation in the inflorescence meristem, which results in increased grain number and yield in rice [2]. *GAD1* encodes a small signal peptide, and loss of function in *GAD1* leads to increases grain number and grain length in cultivated rice. It was speculated that *GAD1* activates *DST* and *CKX2* to degrade the phytohormone cytokinin, and results in reduced grain number in wild rice [3]. Auxin is another key plant hormone in rice panicle development. The *PAY1* gene improves secondary branching, grains per panicle, and grain yield per plant, by influencing the activity of polar auxin transport and altering distribution of indole-3-acetic acid [4]. In recent years more attention was concentrated on BR research in rice. *GNS4*, a novel allele of *DWARF11*, significantly enhances grain number and grain size by regulating the expression levels of BR synthesis and BR response related genes [5]. *MRG702* also regulates grains per panicle and grain shape by affecting BR biosynthesis and signal transduction [6]. *OsRLCK57* interacts with *OsBRI1* (a rice BR receptor) and negatively regulates BR signaling, which affects rice panicle secondary branching [7].

Among cloned rice panicle trait genes, some show pleiotropic effects on tiller or heading date and plant height. In some cases, tillers and panicle branches are consistently regulated by a single gene. *MONOCULM 1* (*MOC1*) is an important example in rice, the *moc1* mutant plants have fewer tillers and panicle branches [8]. Similarly, *LAX1* regulates both tillers and panicle branches in coordination [9]. However, in some cases, the number of tillers or panicle branches changes in the opposite way. For example, *IPA1* (*Ideal Plant Architecture 1*) encodes *OsSPL14* and is regulated by *OsmiR156*. *OsSPL14* mutation perturbs *OsmiR156* direct regulation of *OsSPL14*, which results in reduced tiller number and increased panicle branches [10]. This is similar to *PAY1*, where the *PAY1* mutant displays reduced tiller numbers and increased panicle branches [4]. These findings suggest that rice tillers and panicle branches may be regulated by distinct mechanisms. Some grain number regulation genes demonstrate pleiotropic roles in regulating heading date and plant height. *Ghd7* encodes a CCT domain protein, and has a significance effect on grain number. By regulating *Ehd1* and *Hd3a*, *Ghd7* affects heading date under long-day conditions. *Ghd7* can also enhance plant height by increasing cell numbers [11]. *Ghd8*/*DTH8* and *Ghd7.1*/*DTH7* also have pleiotropic effects on grain number, heading date, and plant height [12–15].

*OsGRF4*, an important pleiotropic gene can significantly enhance rice yield, and was recently cloned. *OsGRF4* encodes the growth-regulating factor 4, a transcription activator, which improve grain size, panicle length, and seed shattering. A rare nucleobase polymorphism in *OsGRF4* in the target site of *OsmiRNA396* is correlated to high expression of *OsGRF4*, which leads to the phenotypic changes in panicle traits [16–18]. *OsGSK2* negatively regulates transcription activation activity of *OsGRF4* by direct interaction; *OsGRF4* also interacts with GRF-interacting factor 1 (*OsGIF1*), which results in bigger grain [19]. The *OsmiR396c-OsGRF4-OsGIF1* regulatory pattern influences grain shape and improves rice yield [20]. Field studies show that *OsGRF4* can increase yield of hybrid rice (13.7%–28.0% increase) [16, 20]. Despite this, the molecular mechanisms of *OsGRF4* in regulating panicle traits remain elusive.

RNA sequencing technology (RNA-Seq) is a promising means for genome-wide transcriptomic analysis. Recently, RNA-Seq has been widely applied to transcriptional analyses associated with rice fertility, heterosis, drought tolerance, salinity stress, heat stress, cold tolerance and biotic stress [21–24]. In consideration of *OsGRF4* is a notable panicle trait gene and has broad application prospects in rice breeding. In this study, to illuminate gene expression regulatory networks involved in panicle trait development, we performed a comprehensive transcriptomic analysis between NIL-*OsGRF4* and NIL-*Osgrf4* in young panicles that were 7 cm

long using RNA-seq. We detected over 331 million raw reads, of which roughly 317 million reads were clean. The error rate of all samples was very low (0.02%). In total, we identified 80 significant differentially expressed genes (DEGs). Hierarchical Cluster analysis showed that *OsGRF4* was tightly clustered with the up-regulated DEG LOC_Os02g47320, those two genes have highly similar gene expression pattern and LOC_Os02g47320 may play a crucial part in panicle trait regulation. We searched the reported functions of DEGs and compared them with previous identified quantitative trait loci (QTL) influencing panicle traits and stress tolerance. Out result suggest that the pleiotropic gene *OsGRF4* may be involved in abiotic resistance. The novel candidate genes identified here may play a significant role in panicle traits regulation, and our results provide valuable information for understanding the molecular mechanisms underlying rice panicle trait development.

## Materials and methods

### Plant materials

The big-grain rice variety CDL (contains *OsGRF4*) was crossed with the medium-grain variety R1126 (contains *Osgrf4*, an allele of *OsGRF4*) to obtain $F_1$ plants, and $F_{10}$ population of recombined inbred lines (RIL) were obtained by single-seed descent. Grain shape difference was observed in one $F_{10}$ line (L28), and L28 showing heterozygous at *OsGRF4* locus was selfed to yield NIL-*OsGRF4* and NIL-*Osgrf4*.

### RNA isolation

Young panicles 7 cm in length were collected from NIL-*OsGRF4* and NIL-*Osgrf4* for RNA isolation and total RNA was extracted using TRlzol Reagent according to the manual instruction (Life technologies, California, USA). Each sample had three biological replicates. The concentration and purity of each RNA sample were assessed by gel electrophoresis and a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). RNA was used for RNA-seq and qRT-PCR.

### Tanscriptome sequencing

Sequence libraries were constructed by the Novogene Bioinformatics Institute (Beijing, China) and sequenced using the Illumina HiSeq™ 2000 platform. Low quality reads (unknown nucleotides and adaptor sequences) were filtered to get clean reads. Clean reads were mapped to the reference genome (ftp://ftp.ensemblgenomes.org/pub/release-20/plants/fasta/oryza_indica/dna/) using Tophat v2.0.9 software (Broad Institute, Cambridge, MA, USA).

### Differential expression analysis

The reads numbers mapped to each gene were counted using HTSeq v0.6.1 software, and gene expression levels were calculated using the reads per kb per million reads (RPKM) values. Differential gene expression analysis between NIL-*OsGRF4* and NIL-*Osgrf4* plants was performed by the DESeq R package, filtering DEGs with $|\log2 (\text{Fold Change})| > 1$ and corrected $p$-value (padj) $< 0.05$. Gene ontology (GO) enrichment analysis of DEGs was implemented by the GOseq R package. GO terms with corrected $p$-value $< 0.05$ were considered significantly enriched. KOBAS software was used to test the statistical enrichment of DEGs in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. RNA-seq data were deposited in the National Center for Biotechnology Information Sequence Read Archive under accession number SRP131560.

### RNA-seq validation by quantitative real-time PCR

To validate RNA-seq DEG data, quantitative real-time PCR (qRT-PCR) was conducted on 24 randomly selected genes (12 down regulated and 12 up-regulated DEGs). Total RNA of NIL-*OsGRF4* and NIL-*Osgrf4* was reverse transcribed using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix for quantitative PCR (qPCR) kit (TransGen Biotech, Beijing, China). The relative expression was analyzed from cycle threshold values using the $2^{-\Delta\Delta Ct}$ method with the rice Ubiquitin 5 gene used as a reference, and all of the results had three biological replicates. The 24 DEGs and primer sequences are listed in S1 Table.

## Results

### The effect of *OsGRF4* on panicle traits phenotype of rice

Rice grains of NIL-*OsGRF4* were significant bigger and heavier compared to that of NIL-*Osgrf4*. This difference resulted in an increased storage capacity (Grain weight × Spikelet number per panicle × Tiller number) in NIL-*OsGRF4* plants by 30.37%. *OsGRF4* also positively regulated panicle length, but resulted in lower seed setting percentage. Interestingly, *OsGRF4* also significantly improve shattering degree [18]. The two NILs had similar germination rates just after harvest, but NIL-*OsGRF4* had very low germination after storing at room temperature for one year (S1 Fig). This result suggests that *OsGRF4* may negative regulate germination.

### Whole genome transcriptome profiles of NIL-*Osgrf4* and NIL-*OsGRF4*

The young panicle stage is critical for panicle traits determination. *OsGRF4* had the highest expression levels in young panicles measuring 7 cm long, and the relative expression was different between NIL-*Osgrf4* and NIL-*OsGRF4* [18]. Therefore, we sequenced the transcriptome profiles of 7 cm young panicle from the two NILs. We detected over 331 million raw reads, and obtained roughly 317 million clean reads, accounting for 95.83% of the raw reads. The error rate was low for all samples (0.02%). The average Q20 and Q30 were 96.35% and 91.02%, respectively. After aligning clean reads to the rice *indica* varieties 9311 reference genome, a total of 80.24–81.37% of reads were mapped, 78.39% of mapped reads were unique, and 2.07%–2.65% of reads were mapped to multiple loci (Table 1). Significant correlation was detected among transcriptome data across all biological replicates of both from NIL-*OsGRF4* and NIL-*Osgrf4* with a correlation coefficient greater than 0.97. The correlation analysis also showed that NIL-*OsGRF4* and NIL-*Osgrf4* had similar genetic background (S2 Fig).

### Identification of DEGs between NILs

We detected 24807 and 25370 expressed genes in NIL-*OsGRF4* and NIL-*Osgrf4*, respectively. A total of 24280 genes were common to both genotypes, with 527 and 1090 genes uniquely expressed in NIL-*OsGRF4* and NIL-*Osgrf4*, respectively (S3 Fig). In total, 80 significant DEGs were detected between NIL-*OsGRF4* and NIL-*Osgrf4*, including 23 up-regulated genes and 57 down-regulated genes (Fig 1a; S2 Table). RNA-seq showed the expression level of the target gene *OsGRF4* (LOC_Os02g47280 / BGIOSGA005785) was up-regulated in NIL-*OsGRF4* plant, which agrees with our previously reported qPCR analysis [18]. Hierarchical cluster analysis of DEGs showed that *OsGRF4* was tightly clustered with LOC_Os02g47320 (S4 Fig), showing that these two genes have similar gene expression patterns. NIL-*OsGRF4* and NIL-*Osgrf4* clustered together (Fig 1b), which revealed analogous genetic backgrounds.

**Table 1. Summary of sequencing data generated and mapped to the reference genome.**

| Sample name | N_DLA | N_DLB | N_DLC | N_XLA | N_XLB | N_XLC |
|---|---|---|---|---|---|---|
| Raw reads | 53581034 | 55079100 | 49357768 | 58703882 | 61334078 | 53044476 |
| Clean reads | 51478186 | 52937908 | 47535552 | 56109570 | 58756544 | 50471646 |
| Clean bases | 7.72G | 7.94G | 7.13G | 8.42G | 8.81G | 7.57G |
| Error rate (%) | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| Q20 (%) | 96.17 | 96.46 | 96.44 | 96.36 | 96.3 | 96.38 |
| Q30 (%) | 90.66 | 91.23 | 91.14 | 91.07 | 90.88 | 91.12 |
| GC content (%) | 51.85 | 51.94 | 51.87 | 52.56 | 51.32 | 52.24 |
| Total mapped | 41413972 (80.45%) | 43060233 (81.34%) | 38681326 (81.37%) | 45023405 (80.24%) | 47301997 (80.51%) | 40517078 (80.28%) |
| Multiple mapped | 1086373 (2.11%) | 1346765 (2.54%) | 995857 (2.09%) | 1163241 (2.07%) | 1418520 (2.41%) | 1336574 (2.65%) |
| Uniquely mapped | 40327599 (78.34%) | 41713468 (78.8%) | 37685469 (79.28%) | 43860164 (78.17%) | 45883477 (78.09%) | 39180504 (77.63%) |

N_DL: NIL-*OsGRF4*; N_XL: NIL-*Osgrf4*; A, B and C represents three biological replicates.

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## Expression patterns of DEGs were validated by qRT-PCR

To confirm DEGs identified in our transcriptome analysis, 24 randomly selected DEGs were analyzed by qRT-PCR. The qRT-PCR analysis showed that the 12 up-regulated DEGs (such as LOC_Os02g47320, LOC_Os04g55159 and LOC_Os04g41680, which involved in vacuolar ATPase G subunit, seed storage and chitinase family protein precursor) demonstrated higher



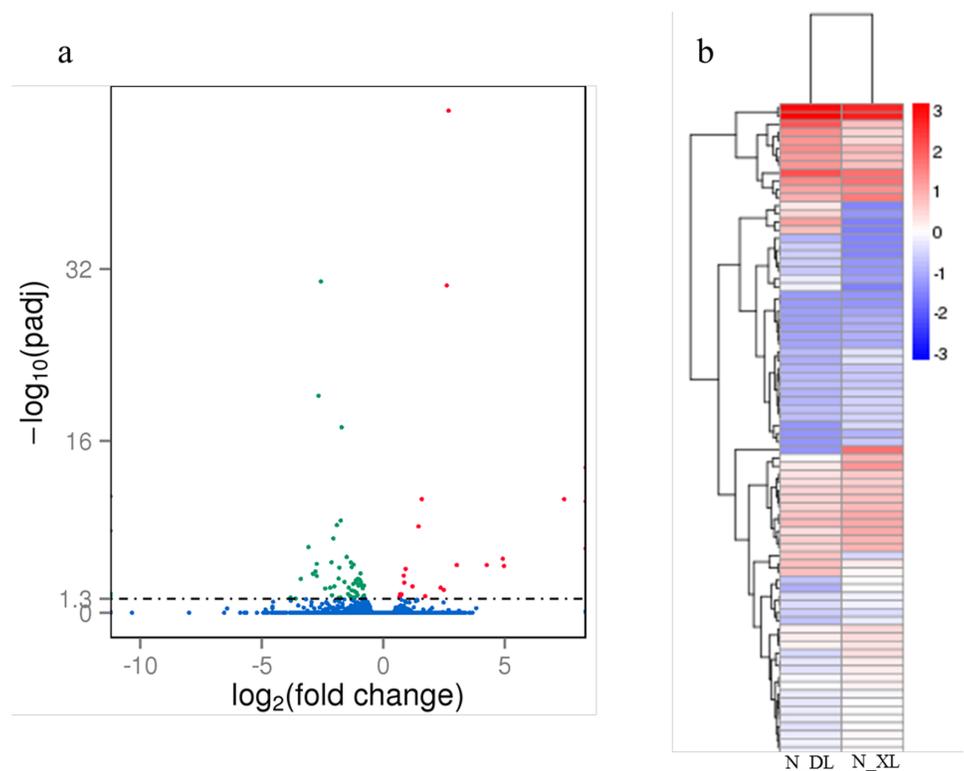**Fig 1. The overall distribution and Hierarchical clustering analysis of differentially expressed genes. (a)** Red and green color represents 23 significant up-regulated DEGs and 57 significant down-regulated DEGs, respectively. **(b)** Red color shows high expression level genes and blue color shows low expression level genes. N_DL: NIL-*OsGRF4*; N_XL: NIL-*Osgrf4*.

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expression levels in NIL-*OsGRF4* than in NIL-*Osgrf4* (Fig 2). The 12 down regulated DEGs (such as LOC_Os05g35500, LOC_Os04g28620 and LOC_Os09g25850, which involved in MYB transcription factor, male sterility and uncharacterised domain Wax2) showed lower expression levels in NIL-*OsGRF4* than in NIL-*Osgrf4*. Expression trends were consistent for all transcripts in qRT-PCR and RNA-Seq analyses.

## Functional classification of DEGs by GO analyzing

Gene ontology analysis of up-regulated genes showed 10 DEGs were enriched in 230 GO terms, including 63 functional terms in molecular function, 46 in cellular components, and 121 in biological processes. Our target gene *OsGRF4* was enriched in 83 functional terms, and over-represented ($P < 0.05$) in 2 GO terms, hydrolase activity (GO:0016787) and macromolecule metabolic process (GO:0043170) (S3 Table). Eight significantly enriched GO terms (corrected $P$-value $< 0.05$) were detected in up-regulated DEGs (Table 2, S3 Table, Fig 3a, S5 and S6 Figs), showing possible biological processes and molecular function underlying panicle trait regulation. Among the down-regulated genes, 36 DEGs were enriched in 296 GO terms. We further identified 47 over-represented GO terms, of which the 5 most highly enriched GO terms were cellulase activity (GO:0008810), cellulose catabolic process (GO:0030245), beta-glucan catabolic process (GO:0051275), glucan catabolic process (GO:0009251) and cellular polysaccharide catabolic process (GO:0044247) (Fig 3b, S4 Table).

## KEGG pathway analysis of DEGs

KEGG analysis showed 10 out of 80 DEGs were enriched across 17 functional categories. Among the up-regulated genes, one showed notable enrichment (corrected $P$-value $< 0.05$) in the pathways for amino sugar and nucleotide sugar metabolism (osa00520), suggesting this pathway may perform an important function in rice panicle development (S5 Table). Among the down-regulated genes, three over-represented KEGG terms ($P < 0.05$) were identified, including cutin, suberine and wax biosynthesis (osa00073), pantothenate and CoA biosynthesis (osa00770), and beta-Alanine metabolism (osa00410) (S6 Table). Subsequent functional analysis of DEGs involved in the KEGG pathway may help elucidate the complex regulatory networks of panicle traits in rice.

## Chromosome co-localization of DEGs with previously identified QTL responsible for panicle traits

To identify the potential function of DEGs, we co-localized previously reported QTLs (http://qtaro.abr.affrc.go.jp/, http://archive.gramene.org/) and DEGs onto rice chromosomes. A total of 54 DEGs (18 up-regulated and 36 down-regulated) were co-localized within 50 QTLs intervals. Among them, 22 QTLs were responsible for grain size or grain weight, 4 for panicle length, 2 for seed shattering, 2 for seed set percent, 9 for seed germination/dormancy, and 11 for cold/drought tolerance. Chromosome 2 had the greatest number of co-localized QTLs with 11 QTLs, followed by chromosome 4 with 8 QTLs (Fig 4, S7 Table).

Three up-regulated DEGs (LOC_Os02g55670, LOC_Os03g26350, and LOC_Os04g53550) co-localized with 3 panicle traits QTLs, respectively. The pleiotropic QTLs *SLCHL4* (*NAL1*) and *OsPTR9* controlling grain weight and panicle size [25, 26] were co-localized with LOC_Os04g53550 and LOC_Os06g50980, respectively. The up-regulated DEG LOC_Os04g41680 belonged to the significantly enriched KEGG pathway (S5 Table) and co-localized with *yd4* responsible for grain yield [27], which suggests that LOC_Os04g41680 may be a candidate panicle trait QTL, playing an important role in the regulatory network of panicle traits by differential expression levels. We also found some DEGs were located in cold/
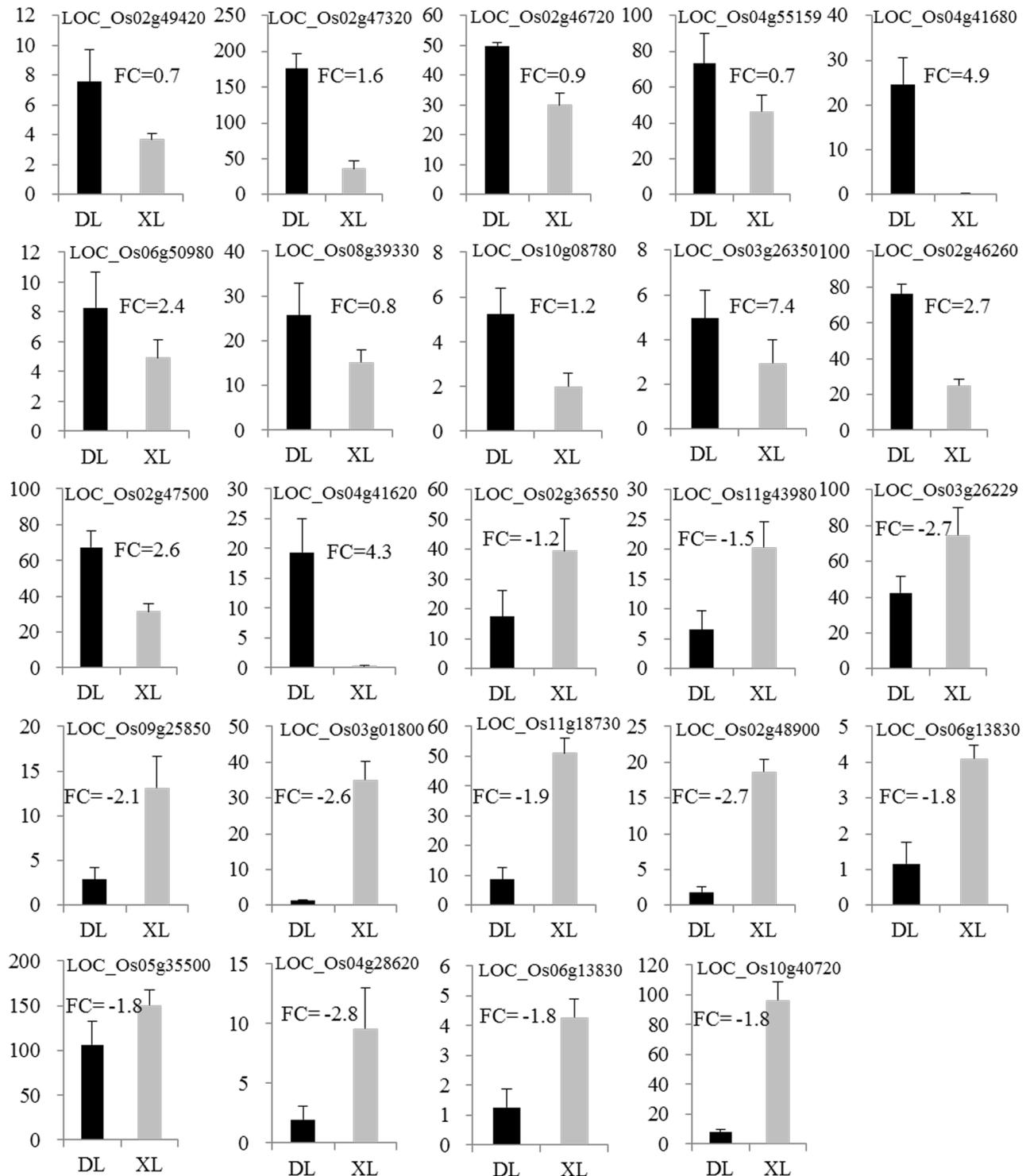
**Fig 2. Expression patterns of DEGs were validated by qRT-PCR.** The relative expressions of 24 DEGs (12 up-regulated genes and 12 down-regulated genes) were analyzed by real-time PCR with 7 cm young panicle. The rice Ubiquitin 5 gene (*OsUBQ5*) was used as a reference, results are showed as means ± SD with three biological replicates. FC: Fold change from transcriptome analysis. DL: NIL-*OsGRF4*; XL: NIL-*Osgrf4*.

**Table 2. The significantly enriched GO terms in the RNA-seq analysis.**

| Term | GO_accession | Sample number | Background number | Corrected *P*-Value |
|---|---|---|---|---|
| Somatic embryogenesis | GO:0010262 | 2 | 10 | 0.011 |
| Chitinase activity | GO:0004568 | 2 | 10 | 0.039 |
| Chitin metabolic process | GO:0006030 | 2 | 10 | 0.039 |
| Chitin catabolic process biosynthesis | GO:0006032 | 2 | 10 | 0.039 |
| Amino sugar catabolic process | GO:0046348 | 2 | 10 | 0.039 |
| Glucosamine-containing compound Metabolic process | GO:1901071 | 2 | 10 | 0.039 |
| Glucosamine-containing compound Catabolic process | GO:1901072 | 2 | 10 | 0.039 |
| Aminoglycan catabolic process | GO:0006026 | 2 | 10 | 0.041 |

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drought tolerance-related QTL regions (Fig 4, S7 Table). For instance, pleiotropic QTLs *OsHsp23.7* and *qLTG-7* were not only responsible for seed germination/dormancy but also for cold/drought and salt tolerance [28, 29]. Interestingly, our target gene *OsGRF4* (LOC_Os02g47280) was located in a cold tolerance QTL *qSDW2* [30] and a drought tolerance QTL *qGY-2b* [31]. This suggests that the pleiotropic gene *OsGRF4* may be involved in abiotic stress resistance. A previous study showed that *OslecRK* was responsible for both plant innate immunity and seed germination, and knocking out *OslecRK* reduced seed germination and resistance to bacterial and fungal pathogens as well as insects in rice [32]. We found a down-regulated gene (LOC_Os05g35500) encoding the Myb transcription factor *Osmyb1*, which may be involved in trans-regulation, was co-localized with 3 QTLs responsible for grain weight (QTL *gw5b* and *gw5.1*) and seed set percent (QTL *ssp-5*) [33–35]. In summary, the co-localized DEGs identified in this study provide a starting point for elucidating the molecular regulation mechanisms of rice panicle traits.

## Discussion

Panicle traits such as panicle shape, grain size, seed germination/dormancy, and seed shattering are important to rice yield and quality. Although a number of genes controlling panicle traits have been isolated, the genetic regulation mechanisms underlying them are still uncertain. Gene expression profiling analysis is a good method for understanding the molecular mechanisms involved in panicle traits regulation. At the same time, current sequencing technologies provide high throughput, accurate, and economical methods to study whole transcriptomes. In recent years, RNA-seq has been widely applied to investigate the transcriptome of numerous plants species across a range of environment conditions, including rice [22], maize [36], sorghum [37], tea plant [38], bamboo [39] and wild species as well [40, 41]. NILs are ideal material for detecting DEGs, having a single gene difference between two genotypes, which eliminates most genetic background noise. For example, Raorane et al analyzed the proteome of spikelets, roots, and flag leaves between NILs with different drought resistant phenotypes and identified DEGs under drought conditions [42]. Similarly, we performed a transcriptomics sequence analysis of young rice panicles between NIL-*Osgrf4* and NIL-*OsGRF4* to study molecular mechanisms of panicle trait regulation. By comprehensively analysis of RNA-seq data, we provide new insights into panicle trait regulation in rice.

Our RNA-Seq analysis identified 80 significant DEGs, including 23 up-regulated DEGs and 57 down-regulated DEGs. The target gene *OsGRF4* (LOC_Os02g47280) was up-regulated according to RNA-Seq results, which is consistent with our previous qPCR results [18]. To evaluate the potential role of identified DEGs, we searched reported functions in existing databases. Eighteen DEGs were reported to be involved in panicle trait regulation in rice. The
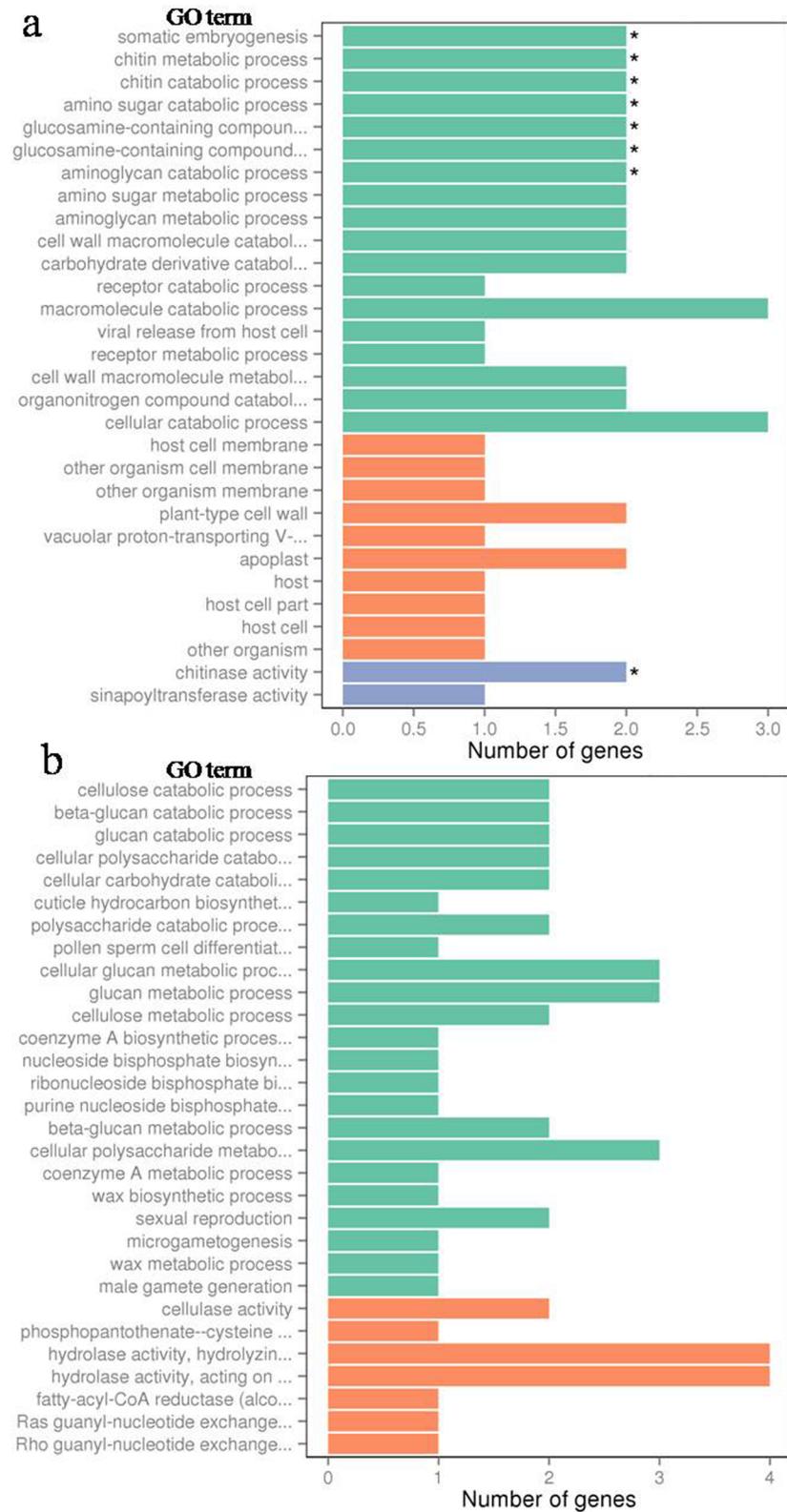
**Fig 3. Gene ontology classifications of DEGs. (a, b)** The most enriched GO categories in up-regulated DEGs and down-regulated DEGs, respectively. * GO terms were significantly enriched. Green, blue and red color represents biological process, molecular function and cellular component, respectively.
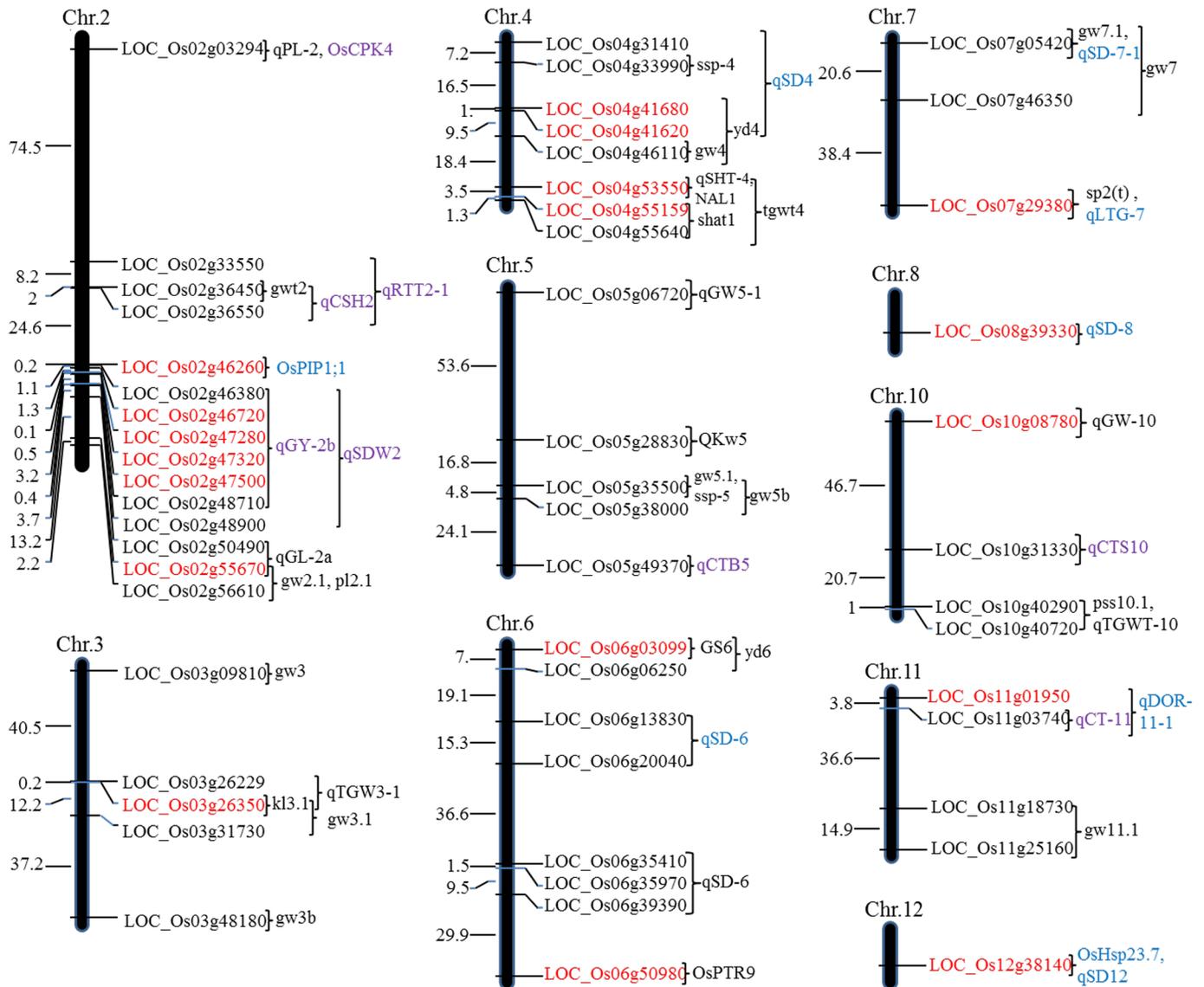
https://doi.org/10.1371/journal.pone.0199077.g003

**Fig 4. The DEGs co-localized with previous identified panicle traits and tolerance QTLs onto rice chromosomes.** For the DEGs, red color represents up-regulated genes, black color represents down-regulated genes; For the QTLs, genes in black, blue and purple letters associated with panicle traits, seed germination/dormancy and cold/drought tolerance, respectively.

down-regulated DEG *BSG1/TH1* (LOC_Os02g56610) encodes a DUF640 domain protein and determines grain width, thickness, and yield by regulating expression levels of cell expansion and division genes [43, 44]. *BSG1/TH1* also co-localized with 100-grain weight QTL *gw2.1* and panicle length QTL *pl2.1* [45]. Rice grain production is also regulated by cytokinin content [2], and we identified four cytokinins significant differences between NIL-*OsGRF4* and NIL-*Osgrf4* [18]. The purine permease family (*OsPUP*) is involved in cytokinin transporter and has 12 members in rice. *OsPUP7* mutants had high cytokinin content, which resulted in larger seeds and increasing sensitivity to salt and drought stresses [46]. The down-regulated DEG *OsPUP11* (LOC_Os02g46380), is also a cytokinin transporter and may influence grain shape. Polyamine is important for normal seed development in Arabidopsis [47] and polyamine oxi-dase (PAO) is a FAD-dependent enzyme associated with polyamine catabolism. The rice

genome has 7 PAOs [48], and of these, the down-regulated DEG *OsPAO2* (LOC_Os03g09810) co-localized with seed weight QTL *gw3* [49], thus, *OsPAO2* may influence grain weight. DEG LOC_Os02g50490 was mainly detected during seed and panicle developmental (stages −5, −6), and therefore may be involved in panicle trait regulation [50].

Transcription factors (TFs) are regulatory proteins and plays a crucial role in panicle trait development [51, 52]. The seed shattering gene *sh4* encodes a nuclear TF that is a member of trihelix TF family, which may be derived from the MYB gene family [53]. Four up-regulated DEGs: LOC_Os02g49420, LOC_Os04g41680, LOC_Os04g41620, and LOC_Os08g39330, are up-regulated by *sh4* [54]. In plants, MYB TFs have been isolated from many species, and serve various functions including involvement in development, primary and secondary metabolism, flowering time, and stresses response [55, 56]. The *Ant28* gene encodes a R2R3 MYB protein which influences barley seed dormancy [57]. *MYB56* encodes a R2R3 MYB TF, which positively controls seed size in *A. thaliana* in an unknown pathway [58]. *Osmyb1* (LOC_Os05g35500) was down-regulated in this study and was found to be co-localized with 3 QTLs which determine grain weight (QTL *gw5b* and *gw5.1*) and seed set percent (and QTL *ssp-5*) [33–35]. Phylogeny analysis showed that *AtMYB66* (encoding the R2R3 MYB protein) had the closest genetic relationship with *Osmyb1* [59]. The expression levels of *Osmyb1* and *Osmyb4* peaked in seeds at 14 days after flowering, which suggests that these two genes may function in seed maturation [60]. Other studies have shown that *Osmyb1* is associated with cold and drought tolerance [61, 62]. Therefore, the *Osmyb1* TF may act as a key regulator of panicle traits and abiotic resistance.

The pleiotropic gene *OsGRF4* may also affect other phenotype besides panicle trait. It was reported that *OsGRF4* (LOC_Os02g47280) was a target gene of *osa-MIR396c*, and overexpression of *osa-MIR396c* was shown to reduce alkali and salt stress tolerance. *OsGRF4* was up-regulated when treated by saline, anoxia, ABA, and gibberellins, and down-regulated by arsenate [63]. Another study found that LOC_Os02g47280 showed differential expression in root knot nematode infection [64]. Interestingly, we found that *OsGRF4* co-localized with both a cold tolerance QTL (*qSDW2*) [30] and a drought tolerance QTL (*qGY-2b*) [31]. Many DEGs detected in this study, such as LOC_Os04g55159, LOC_Os04g41680, LOC_Os04g41620, LOC_Os04g53550, *OsGL1-1* (LOC_Os09g25850), LOC_Os10g31330 and LOC_Os04g33990, were found to be regulated by biotic and abiotic stress such as cold, salt, and drought stress as well as blast disease infection [65–69]. The *Osmyb1* TF not only influences seed maturation [60], but was also found to be associated with cold and drought tolerance [61, 62]. Therefore, *OsGRF4* and some DEGs detected in this study may be involved in abiotic and biotic stress responses, which need further investigation.

In our GO enrichment analysis, two up-regulated DEGs (LOC_Os04g41680 and LOC_Os04g41620) were significantly enriched in the top 8 GO terms, and were Over_represented in term of seed development (GO: 0048316) (S3 Table). Therefore, LOC_Os04g41680 and LOC_Os04g41620 may be important genes regulating grain shape. Along with LOC_Os04g41680 and LOC_Os04g41620, three up-regulated genes (*OsGRF4*, LOC_Os02g47320, and LOC_Os02g46260) and 9 down-regulated genes (LOC_Os02g50490, LOC_Os03g01800, LOC_Os02g48900, LOC_Os12g04320, LOC_Os10g05910, LOC_Os06g13830, LOC_Os06g06250, LOC_Os05g06720, and LOC_Os07g46350) were enriched for hydrolase activity (GO: 0016787) (S3 and S4 Tables). *TGW6* encodes a novel protein that has indole-3-acetic acid-glucose hydrolase activity, and enhances rice seed weight and yield [70]. Therefore, DEGs enriched in hydrolase activity GO term may be potential candidate genes for grain shape. *OsGRF4* and LOC_Os02g47320 were collectively enriched in 14 GO terms (S3 Table), and LOC_Os02g47320 was also involved in 3 KEGG pathways (S5 Table). In addition, *OsGRF4* was tightly clustered with LOC_Os02g47320 (S4 Fig), which shows that these two genes have

highly similar gene expression pattern and LOC_Os02g47320 may play a crucial role in panicle trait regulation, however further research is needed.

In summary, our transcriptome sequence analyses provide some important candidate genes involving in panicle trait regulation, which may be used in future gene cloning studies. Functional analysis of the DEGs and pathways identified here will serve to further our understanding of the transcriptional network and molecular mechanisms underlying panicle trait regulation in rice.

## Supporting information

**S1 Fig. Seed germination percentage of NIL-*OsGRF4* and NIL-*Osgrf4*.** The seeds have been stored at room temperature over one year.
(TIF)

**S2 Fig. Pearson correlation coefficient between samples.** N_DL: NIL-*OsGRF4*; N_XL: NIL-*Osgrf4*; A, B and C represents three biological replicates.
(TIF)

**S3 Fig. Venn diagram showing the genes expressed in NIL-*OsGRF4* and NIL-*Osgrf4*.** A total of 24807 and 25370 genes with expression were identified from NIL-*OsGRF4* and NIL-*Osgrf4*, respectively. N_DL: NIL-*OsGRF4*; N_XL: NIL-*Osgrf4*.
(TIF)

**S4 Fig. Cluster analysis of differentially expressed genes.** *OsGRF4* (BGIOSGA005785) tightly clustered together with LOC_Os02g47320 (BGIOSGA005784). N_DL: NIL-*OsGRF4*; N_XL: NIL-*Osgrf4*.
(TIF)

**S5 Fig. GO analysis of differentially expressed genes of the biological process category.**
(TIF)

**S6 Fig. GO analysis of differentially expressed genes of the molecular function category.**
(TIF)

**S1 Table. qRT-PCR primer sequence.**
(XLSX)

**S2 Table. A total of 80 differentially expressed genes identified between NIL-*OsGRF4* and NIL-*Osgrf4*.**
(XLSX)

**S3 Table. GO enrichment analysis for the up-regulated genes.**
(XLSX)

**S4 Table. GO enrichment analysis for the down-regulated genes.**
(XLSX)

**S5 Table. NIL-*OsGRF4* vs NIL-*Osgrf4* up-regulated DEGs KEGG_pathway.**
(XLSX)

**S6 Table. NIL-*OsGRF4* vs NIL-*Osgrf4* down-regulated DEGs KEGG_pathway.**
(XLSX)

**S7 Table. List of previously identified panicle traits and tolerance QTLs downloaded from QTARO and gramene database.**
(XLSX)

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