1. Introduction

The growth and development of flowers underpins the development of angiosperm seeds. Rice (Oryza sativa) is a monocotyledonous model plant used in the study of angiosperm flower development, and has a unique inflorescence (panicle) architecture composed of several spikelets [1–4]. Each rice spikelet consists of a terminal fertile floret, which comprises one lemma, one palea, two lodicules, six stamens, and one pistil, in addition to two pairs of sterile glumes known as the rudimentary glumes and sterile lemmas [5–8].

Recently, several genes have been reported to be associated with the growth and development of the sterile lemmas and rudimentary glumes in rice, including LONG STERILE LEMA (G1) [9], OsMADS34 [10–12], EXTRA GLUME 1 (EG1) [13], FRIZZY PANICLE (FZP) [14,15], SUPERNUMERARY BRACHT (SNB) [16,17], INDETERMINATE SPIKELET 1 (IDS1) [17], MULTI-FLORET SPIKELET 1 (MFS1) [18], and FLORAL ORGAN NUMBER 4 (FON4) [19]. G1 encodes a plant-specific protein that determines the identities of the sterile lemmas. In the g1 mutant, the sterile lemmas are transformed into lemma-like organs [9]. OsMADS34 is a MADS-box transcription factor, and the loss of its function causes the sterile lemmas and rudimentary glumes to develop a lemma-like cellular structure [10–12]. EG1 encodes a putative triacylglycerol lipase protein that affects the sterile lemma fate and floral meristem determinacy. The eg1 mutant has elongated sterile lemmas and ectopic floral organs [13]. SNB and FZP determine the identity of the sterile lemmas and rudimentary glumes, with the snb and fzp mutants typically displaying additional rudimentary glumes but lacking the sterile lemmas [15–17]; however, the fzp-12 mutant produced smaller sterile lemmas and showed no changes in its rudimentary glumes [14]. IDS1 and MFS1 also regulate the development of sterile lemmas, which were transformed into the rudimentary glumes in the ids1 and mfs1 mutants [17,18]. The above studies revealed that the lemma, sterile lemmas, and rudimentary glumes may be homologous organs; however, the origins and evolution of the sterile lemmas and rudimentary glumes are not completely clear.
Increasing the grain yield of rice, a major cereal crop, is an important focus of current research. Rice yield is the product of many factors, including the panicle numbers per plant, grain numbers per panicle, and the grain weight [14,20–22], with grain size being one of the most important factors determining grain weight and thus rice yield [23,24]. So far, several genes regulating grain size have been isolated and studied, including Grain Length and Weight on chromosome 7 (GLW7), Grain Size 3 (GS3), and Grain Length 3 (GL3), all of which regulate grain length; and Grain Width and Weight 2 (GW2), GW5, GW8, and GS5, which influence grain width. GLW7 encodes a transcription factor, SPL13, which positively regulates grain size by increasing cell size [25]. GS3 is a negative regulator of grain size and encodes a putative transmembrane protein [26,27]. GL3 encodes a phosphatase kelch protein, which enhances grain length by increasing the number of cells [28]. GW2 encodes a RING-type E3 ubiquitin ligase, the loss of which increases grain widths by increasing the number of cells in the spikelet hull [29]. GW5 is a calmodulin-binding protein that determines grain width and weight by positively regulating brassinosteroid signaling [30], while GW8 encodes an SBP-domain transcription factor, SPL16, that regulates grain size by promoting cell proliferation [31,32]. GS3 encodes a putative serine carboxypeptidase and functions as a positive regulator of grain size [33]. Additionally, Grain Length on chromosome 7 (GL7), GS2, Big Grain 1 (BG1), and BG2 regulate grain length and width [21,34–36]. These findings revealed that several genes have a multi-pathway effect on grain size, and that cell proliferation and expansion play key roles.

In this study, we identified a new rice spikelet mutant, lacking rudimentary glume 1 (lrg1). Using map-based cloning, we isolated the LRG1 gene, which encodes a ZOS4-06-C2H2 zinc-finger protein. The mutation of LRG1 results in the loss of one of the two rudimentary glumes, the elongation of the remaining rudimentary glume and the sterile lemmas, the formation of additional lemmas, and the production of shorter and wider grains. These results suggest that LRG1 is involved in the regulation of the development of the rudimentary glumes and sterile lemmas, spikelet meristem determinacy, and grain size.

2. Materials and methods

2.1. Plant materials

The lrg1 mutant was derived from a mutant population generated by the treatment of the japonica rice cultivar Yunjing (YJ17) with ethyl methanesulfonate (EMS). YJ17 was used as the wild type in this study. The F1 generation was obtained by crossing the lrg1 mutant and Shuhui 527 (indica cultivar), and was grown in Hainan Province, China. The F2 generation was obtained by self-fertilization of the F1 plants, and was planted in Hangzhou, Zhejiang Province, China. The obtained F2 population was used for the map-based cloning of the LRG1 gene.

2.2. Microscopy analysis

For the histological analysis using paraffin sections, the unpollinated spikelets of YJ17 and lrg1 were fixed in 70% formalin-acetic acid-alcohol (FAA) solution, after the samples were processed as described by Yu et al. [37]. The treated paraffin sections were observed using a microscope (Leica DM4 B). Fresh young panicles and seeds at the filling stage were observed on a –25 °C freezing platform using a scanning electron microscope (Hitachi S-3500).

2.3. Map-based cloning of LRG1

A total of 628 plants displaying the lrg1 mutant phenotype were selected from the F2 population derived from a cross between the lrg1 mutant and Shuhui 527. The initial gene mapping was performed using published SSR primers. For the fine mapping of the LRG1, new molecular markers were developed. The primers are shown in Table S1 (online).

2.4. Complementation test

To construct the complementary LRG1-COM vector, a genomic fragment consists of a 2860-bp upstream sequence from the start codon and 525-bp coding region sequence of the LOC_Os04g36650 gene in the wild type, was cloned into the pCAMBIA1301 vector with GFP. The LRG1-COM plasmid was transformed into Agrobacterium tumefaciens (EHA105), then transformed into the lrg1 mutant as described previously [38]. The primers used for the vector construction are shown in Table S1 (online).

2.5. RNA extraction and expression analysis

The total RNAs of the young wild-type and lrg1 panicles and floral organs were extracted using an AxyPrep Multisource Total RNA Miniprep Kit (Axygen). The reverse transcription of the RNA was performed using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo), after which a qPCR analysis was performed on a CFX96 Real-Time System (Bio-Rad Laboratories) using a SYBR Green Realtime PCR Master Mix (Toyobo). At least three biological replications were performed for each sample. The qPCR primers are shown in Table S1 (online).

2.6. In situ hybridization

Young panicles from the wild-type and lrg1 plants were fixed in 70% FAA (RNase free) then dehydrated through a series of alcohols/xylenes, before being finally embedded in paraffin (Sigma-Aldrich). All probes used in the experiment were prepared using the same method and labeled using the DIG RNA Labeling Kit (SP6/T7: Roche). The in situ hybridization was performed as described previously [39]. The primers are shown in Table S1 (online).

2.7. Flow cytometric analysis

Fresh young panicles were soaked in a nuclear separation and staining solution (Beckman) before being cut into small pieces. After the plasma was filtered through a 40-mm nylon filter, the cell nucleus suspension was loaded into a Beckman MoFlo flow cytometer. Each flow cytometry experiment recorded the ploidy of about 10,000 nuclei. These data were calculated using the FCS Express software.

2.8. Subcellular localization analysis

The coding fragment of wild-type LRG1 without the stop codon was inserted into the 35S-GFP (S65T)-NOS (pCAMBIA1301) plasmid, and the constructed vector was named LRG1-GFP. Plasmids containing LRG1-GFP or GFP were transformed into rice protoplasts. After 12 h of culture in the dark at 28 °C, the GFP fluorescence was observed using a confocal microscope (Olympus IX71). The primers are shown in Table S1 (online).

2.9. Transcription activity analysis

The coding sequences of LRG1 or FZP were inserted into the pGBK7 plasmid containing the GAL4 DNA-binding domain. FZP-BD was used as a positive control in the study [14]. LRG1-BD or FZP-BD was transformed into yeast strain AH109, and their transcriptional activity was analyzed using SD/−Trp plates and SD/−Trp/−His/−Ade plates. The primers are shown in Table S1 (online).
2.10. DLR assay

The transcriptional activity of LRG1 was analyzed using a DLR assay system in rice protoplasts. The firefly LUCIFERASE gene was driven by the CaMV 35S promoter, and five copies of the GAL4 binding element were used as the reporter [40]. The coding sequences of LRG1, FZP, LRG1 with FZP, and LRG1 with VP16 were independently inserted into the GAL4 binding domain. GAL-BD-FZP and VP16 were used as a positive control. These constructed vectors were transformed into rice protoplasts and cultured in the dark at 28 °C for 14 h, after which the relative luciferase activity was measured using a GloMax 20–20 luminometer (Promega). The primers are shown in Table S1 (online).

2.11. Yeast two-hybrid assay

The coding sequence of LRG1 was inserted into the pGADT7 plasmid, while those of TPR1, TPR2, or TPR3 were inserted into the pGBK7 plasmid. AD-LRG1 was co-transformed into the yeast strain AH109 alongside BD-TPR1, BD-TPR2, BD-TPR3, or pGBK7. The yeast cells were then cultured on SD/-Trp/-Leu plates and SD/-Ade/-His/-Trp/-Leu plates. The primers are shown in Table S1 (online).

2.12. Pull-down assay

The coding sequence of LRG1 was inserted into the pMAL-c2x vector to construct the plasmid MBP-LRG1. The coding regions of TPR1, TPR2, and TPR3 were independently inserted into the pGEX4T-1 vector to construct GST-TPR1, GST-TPR2, and GST-TPR3, respectively. These constructed plasmids were transformed into Escherichia coli BL21 competent cells, and IPTG was used to induce the production of the proteins. The corresponding amylose and GST-binding resins were used for the purification of MBP and GST from the E. coli lysates, respectively. For the in vitro GST pull-down assay, GST or GST-fusion proteins coupled to Glutathione Sepharose 4 fast flow beads were incubated with MBP or MBP-LRG1 at 4 °C for 30 min. The MBP and GST-fusion proteins were detected using anti-MBP and anti-GST antibodies, respectively. The primers are shown in Table S1 (online).

3. Results

3.1. The lrg1 mutant shows defects in spikelet development

In wild-type rice, each spikelet consists of one terminal floret and two pairs of lateral organs: the sterile lemmas and the rudimentary glumes (Fig. 1a–g). By contrast, the lrg1 mutant produced defective rudimentary glumes and sterile lemmas, although no abnormalities were found in the inner whorls of the floral organs (Fig. 1h–n). In the wild-type spikelet, the two rudimentary glumes and two sterile lemmas were glume-like and shorter than the lemma and the palea, while the lrg1 spikelet lacked one rudimentary glume, resulting in the presence of only three glume-like organs, all of which were elongated to lengths close to that of the lemma and palea (Fig. 1a–c and h–j). Some lrg1 spikelets developed an extra lemma-like organ, resembling the lemma in size (Fig. 1o–u).

To further investigate the morphological differences between these structures, we examined the histological characteristics of the elongated glumes and the additional lemma-like organ in the lrg1 mutant (Figs. 1k–n, r–u, S1a–l and S2b online). In the wild-type spikelet, each spikelet consists of one terminal floret and two pairs of lateral organs: the sterile lemmas and the rudimentary glumes. By contrast, the lrg1 mutant produced defective rudimentary glumes and sterile lemmas, although no abnormalities were found in the inner whorls of the floral organs. In the wild-type spikelet, the two rudimentary glumes and two sterile lemmas were glume-like and shorter than the lemma and the palea, while the lrg1 spikelet lacked one rudimentary glume, resulting in the presence of only three glume-like organs, all of which were elongated to lengths close to that of the lemma and palea. Some lrg1 spikelets developed an extra lemma-like organ, resembling the lemma in size.
type, the lemma contained five vascular bundles and its edges were hooked inwards (Fig. 1d and e). The epidermal cells of the lemma were arranged regularly and formed many protrusions and trichomes (Fig. S1a online). The wild-type palea had three vascular bundles and was composed of two parts: the body of the palea (bop) and the two marginal regions of the palea (mrp) (Figs. 1d, e and S1b online). In addition, the wild-type sterile lemmas only contained one vascular bundle, while no vascular bundles were found in the rudimentary glumes (Fig. 1f and g). The sterile lemma had a smooth surface with only rare trichomes on its margins, while the surface of the rudimentary glumes was rough and contained numerous protrusions and trichomes (Fig. S1c and d online). By contrast, the elongated sterile lemmas and the extra lemma-like organs of the lrg1 mutant contained four or five vascular bundles, developed inwardly hooked margins, and bore protrusions and trichomes on its surface, similar to that of the wild-type lemma (Figs. 1l, m, s–u and S1g, j, k online). The elongated rudimentary glume of the lrg1 mutant contained one or two vascular bundles and bore a smooth surface, resembling the wild-type sterile lemma (Figs. 1n, S1h, l and S2a online).

Next, we used the floral identity genes to investigate the identities of the defective and additional organs in the lrg1 mutant. OsMADS1, OsMADS14, OsMADS15, and DL were expressed in the elongated sterile lemmas and the additional lemmas, while no OsMADS6 signals were detected in these organs (Fig. S1m online). These results indicated that the elongated sterile lemma and the additional lemma-like organ acquired a lemma identity, whereas the elongated rudimentary glume had a identity of sterile lemma or lemma.

3.2. The lrg1 mutant exhibited abnormal early spikelet development

To elucidate the process of abnormal spikelet development in the lrg1 mutant, we monitored the young panicles in this line and the wild type at different developmental stages using scanning electron microscopy (SEM) (Fig. S3a–l online). During spikelet development stage 4 (Sp4), the wild-type lemma and palea primordia began to develop, the wild-type sterile lemma and rudimentary glume continued to grow, and the sterile lemma was observed to be longer than the rudimentary glume (Fig. S3a online). In the lrg1 mutant, the sterile lemmas were similar in morphology to the lemma and palea, with more overlap in their edge parts (Fig. S3e online). Only one rudimentary glume was observed in the lrg1 spikelets, which was similar in size and morphology to the sterile lemma (Fig. S3e online). At the Sp5 to Sp6 stages, the wild-type spikelet formed six stamen primordia around the apex floral meristem, the growth of the rudimentary glume was terminated, while the sterile lemma continued to elongate (Fig. S3b online). In the lrg1 mutant, both the sterile lemma and the rudimentary glume continued to develop, with no differences observed in their morphology or size (Fig. S3f online). During the Sp7 and Sp8 stages (the formation of the pistil primordia), the sterile lemma of the wild-type spikelet was much smaller than the lemma (Fig. S3c and d online). In the lrg1 mutant, the sterile lemma continued to enlarge and was almost half the size of the lemma (Fig. S3g and h online). The rudimentary glume of the lrg1 mutant also continued to elongate and was not distinguishable from the sterile lemma (Fig. S3g and h online). During the Sp4 to Sp8 stages, some lrg1 spikelets produced an extra lemma-like organ, which resembled the true lemma at each developmental stage (Fig. S3i–l online). These defects throughout spikelet development were consistent with the defects observed in the mature spikelets at the heading stage.

3.3. The lrg1 mutant produces short, wide grains

The grains of the lrg1 mutant were significantly altered in size (Fig. 2a–v). In the wild type, the average lengths of the grains...
and brown grains were 9.3 and 6.6 mm, respectively, while the lrg1 grains and brown grains averaged 7.2 and 5.3 mm in length, respectively (Fig. 2r, u). Despite being shorter, the lrg1 grains were wider than those of the wild type; in the wild type, the average widths of the grains and brown grains were 3.05 and 2.45 mm, respectively, while the average widths of the lrg1 grains and brown grains were 3.26 and 2.57 mm, respectively (Fig. 2s, v). Furthermore, the thousand-grain and thousand-brown-grain weights of the lrg1 mutant were markedly decreased compared with those of the wild type (Fig. 2q, t). These results indicated that the mutation of LRG1 altered the grain size, thereby affecting the grain yield.

3.4. LRG1 influences grain size by regulating cell division and expansion in the spikelet hull

To reveal how LRG1 regulates grain size at the cellular level, we analyzed the cell sizes and numbers in the lrg1 and wild-type spikelet hulls using paraffin sectioning and SEM (Figs. 2c–f, i–l and S2c, d online). By observing a longitudinal section of the hull, we found that the cell number along the longitudinal axis was significantly reduced in the lrg1 mutant compared with that of the wild type (Fig. 2m). In the outer epidermal layer of the hull, the lrg1 cells were significantly shorter than those of the wild type (Fig. 2n). Consistent with this, the cells of the inner epidermal layer of the lrg1 hull were also significantly shorter than those of the wild type (Fig. S2c–e online). Meantime, no significant differences were observed in the cell widths in the outer and inner epidermal layers of the hull between the wild type and the lrg1 mutant. However, the total and average cell lengths of the lrg1 hull cross section were significantly increased compared with the wild type (Fig. 2o and p). These results indicated that the shorter grain phenotype in the lrg1 mutant may result from a decrease in cell number and length on the longitudinal axis, while their greater widths may be attributed to an increase in cell size in the hull cross section.

To further investigate the effects of LRG1 on cell proliferation and expansion in the spikelet hulls, we performed a quantitative analysis of the expression of several genes known to affect grain size by regulating cell proliferation and expansion. Compared with the wild type, the expression levels of BG1, BG2, and GS3 were dramatically increased in the lrg1 mutant, while GS2 and GS5 were markedly reduced in the mutant (Fig. S2f online). In addition, we also analyzed the expression levels of several genes that directly regulate the cell cycle and cell expansion. The expression levels of CYC2.1, CYCA2.1, CYCA2.2, CYCA2.3, CYC3D, CYC4, CYC7, H1, and MAD2 were markedly lower in the lrg1 mutant than in the wild type (Fig. S4a online). The expression of the cell expansion gene EXP1 was also significantly reduced, while the expression of another expansion-related gene, EXP25, was increased in the lrg1 mutant relative to the wild type (Fig. S4a online).

We detected the cell cycle phase in the young lrg1 panicles using flow cytometry (Fig. S4b–d online). The percentage of G2/M-phase cells with a high 4C DNA content was significantly decreased in the lrg1 mutant relative to the wild type, while the percentage of G1-phase cells with a high 2C DNA content was significantly increased (Fig. S4d online). These results indicate that the cell proliferation rate is slowed in the lrg1 mutant during panicle development. Taken together, these data provide evidence that LRG1 is involved in the regulation of cell proliferation and expansion in the spikelet hull, thereby influencing grain size.

3.5. Mapping and identification of the LRG1 gene

In order to clone the LRG1 gene, we crossed the lrg1 mutant with Yj17. All of the F1 plants exhibited wild-type phenotypes, while the F2 generation showed a 3:1 wild-type:mutant segregation ratio. We selected 128 primer pairs targeting polymorphisms between the parents from 312 pairs of simple sequence repeats (SSR) primers that were evenly distributed across the 12 rice chromosomes. These polymorphic primers were used to screen DNA pools containing 20 wild-type individuals or 20 lrg1 individuals. We found that primers M3 and M5 on chromosome 4 revealed polymorphisms between the wild-type and mutant gene pools. Furthermore, 94 F2 recessive individuals were used for a linkage analysis, and the mutated locus was preliminarily identified as being located between the SSR markers M3 and M5 on chromosome 4 (Fig. 3a).

For the fine mapping, new SSR markers were developed between M3 and M5. Eight markers (M6, M9, M13, M16, M20, M21, M25, M26) exhibited polymorphisms between the two parents, and we used to analyze all 628 F2 recessive individuals to narrow the region containing the LRG1 gene. The LRG1 gene was found to be located between markers M21 and M26 on chromosome 4, which corresponded to an approximately 19-kb physical region in the Nipponbare genome (Fig. 3a). By sequencing all the predicted genes within this target region, we found three mutations (G to A, and AG to TT) within a gene encoding a ZOS4-06-C2H2 zinc-finger protein (LOC_Os04g36650). These mutations caused two amino acid substitutions (Gly-52 to Ser-52 and Gln-54 to Leu-54) in the lrg1 mutant (Fig. 3a). To confirm that the mutation of LOC_Os04g36650 led to the lrg1 mutant phenotypes, a fragment consists of a 2860-bp upstream sequence from the start codon and 525-bp coding region sequence of the LOC_Os04g36650 gene in the wild type, was cloned into the pCAMBIA1301 vector with green fluorescent protein (GFP), and transformed into the lrg1 seeds. Among all the transgenic lines, four lines showed a wild-type phenotype, indicating that the lrg1 mutant phenotype was completely rescued (Fig. 3b–m). We therefore determined that LRG1 is LOC_Os04g36650, and that its mutation resulted in the lrg1 mutant phenotype.

3.6. LRG1 expression pattern analysis

We used quantitative real-time PCR (qRT-PCR) to investigate the expression of LRG1, revealing that it was expressed in all tested organs and tissues, including the roots, culms, leaves, panicles at different developmental stages, rudimentary glumes, sterile lemmas, and all four floral organ whorls (Fig. 4a). The LRG1 transcripts were more abundant in the inflorescences than in the vegetative organs, and increased gradually as the development of the inflorescences progressed (Fig. 4a). The high expressions in the inflorescences were consistent with its function in regulating grain size and spikelet development. However, the low expressions in the vegetative organs implied that LRG1 and other genes may redundantly regulate the development of these organs. In the transgenic complementary plants, strong GFP signals were detected in the rudimentary glumes and sterile lemmas (Fig. 4b).

Furthermore, we also further examined the temporal and spatial expression of LRG1 using in situ hybridization. At the Sp4 stage, the LRG1 transcript signals were observed in the floral meristem (Fig. 4c). At stages Sp5 and Sp6, LRG1 was highly expressed in the primordia of the rudimentary glumes and the sterile lemmas (Fig. 4d). At stages Sp7 and Sp8, the LRG1 transcripts were only found in the rudimentary glume (Fig. 4e and f). These results indicated that LRG1 was mainly expressed in the rudimentary glume during spikelet growth and development, and that its expression in the sterile lemma decreased gradually as spikelet development progressed.

3.7. LRG1 encodes a ZOS4-06-C2H2 zinc-finger protein

A phylogenetic tree analysis revealed that LRG1 and its orthologs from various dicot and grass species formed a LRG1-like clade,
with rice LRG1 belonging to the grass subclade. A protein sequence analysis showed that all LRG1-like proteins had a highly conserved C2H2 zinc-finger domain and an ethylene response factor-associated amphiphilic repression (EAR) motif belonging to the LxLxL subclass (Fig. 4g) [41]. The EAR motif was located at the C-terminus of all proteins amino acid sequences, with the exception of GRMZM2G703281 (\textit{Zea mays}), which only contained a C2H2 zinc-finger domain (Fig. S5 online). The conserved amino acid sequences observed in these proteins could represent the basic functional domain of the C2H2 zinc-finger protein (Fig. S5 online).

LRG1, Bradi5g11180 (\textit{Brachypodium distachyon}), Brast09G094100 (\textit{Brachypodium stacei}), Sobic.006G092300 (\textit{Sorghum bicolor}), GRMZM2G703281 (\textit{Zea mays}), GRMZM2G074793 (\textit{Zea mays}), and Os02g0562200 (\textit{Oryza sativa}) from the LRG1-like grass subclade shared two EAR motifs, which were located in their N-terminal and C-terminal regions. These results implied that the LRG1-like genes from the grasses may have different evolutionary functions to those in the dicots.

To investigate the subcellular localization of the LRG1 protein, the vector containing the full-length LRG1 protein fused to the GFP reporters were transiently expressed in tobacco (\textit{Nicotiana tabacum}) epidermal cells and rice protoplasts, respectively. The fluorescence signals of plants only expressing GFP were ubiquitously detected in the rice protoplasts and tobacco cells, respectively, whereas the fluorescence signals of the LRG1-GFP fusion protein in the rice protoplasts and the LRG1–GFP fusion protein in the tobacco cells were visible only in the nucleus (Fig. 4h–q).

3.8. LRG1 transcriptional activity analysis

LRG1 is thought to be a novel transcription factor containing a zinc-finger domain. To clarify whether LRG1 has transcriptional activity, we performed yeast and dual luciferase reporter (DLR) assays. First, we fused the coding region of LRG1 or the well-known transcriptional activator FZP to the GAL4 DNA-binding domain (BD). FZP-BD was used as a positive control, while the empty pGBKT7 vector was used as a negative control [14]. The constructed LRG1-BD, FZP-BD, and empty pGBKT7 vectors were transformed into yeast competent cells. The yeast cells containing LRG1-BD or the empty pGBKT7 vector could not survive in a culture medium that lacked histidine, tryptophan, and adenine (SD/-Trp/-His/-Ade), whereas the yeast cells transformed with FZP-BD could grow normally (Fig. S6a and b online), suggesting that LRG1 had no transcriptional activation activity.

Subsequently, we further analyzed the transcriptional activity of LRG1 using a DLR assay. The coding frames of LRG1, FZP, LRG1–FZP, VP16, and LRG1–VP16 were individually fused into the GAL vector under the control of the 35S promoter from the Cauliflower mosaic virus (CaMV). GALBD–FZP and VP16 proteins showed higher transcriptional activity than those of the empty GAL vector, suggesting that LRG1 had no transcriptional activation activity.

Supporting Information

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Fig. 3. (Color online) Map-based cloning of the mutated gene. (a) Map position of the LRG1 locus. (b) Wild type spikelet. (c) The lrg1 spikelet with the elongated sterile lemmas and elongated rudimentary glume. (d) The lrg1 spikelet with the extra lemma, the elongated sterile lemmas and elongated rudimentary glume. (e) Defective spikelets of lrg1 mutant were rescued in the transgenic complementation plants. (f) Mature grains of the wild type, lrg1 mutant and the transgenic complementation plant from left to right. (g) Brown rice of the wild type, lrg1 mutant and the transgenic complementation plant from left to right. (h)–(m) Size and weight of grain and brown grain in the wild type, lrg1 mutant and the complementation plant. el, extra lemma-like organ; erg, elongated rudimentary glume; esl, elongated sterile lemma; le, lemma; pa, palea; sl, sterile lemma. Bars = 5 mm in (b)–(g). Error bars mean S.D. **P < 0.01.
activity, while GALBD-LRG1 protein showed lower transcriptional activity (Fig. S6c online). Concurrently, we also found that the transcriptional activity of GALBD-LRG1-FZP and GALBD-LRG1-VP16 proteins were significantly reduced in comparison with GALBD-FZP and VP16, respectively, suggesting that LRG1 repressed the expression of FZP and VP16 (Fig. S6c online). These results indicated that LRG1 possesses transcriptional inhibitor activity.

3.9. LRG1 affects the development of the rudimentary glumes and sterile lemmas by regulating the expression levels of G1, DL, OsMADS1, and OsMADS6

To reveal the mechanism by which LRG1 regulates the development of the lateral organs, we examined the spatial expression patterns of some floral organ recognition genes (G1, DL, OsMADS1, and OsMADS6) during spikelet growth and development using qRT-PCR and in situ hybridization (Figs. 5a–r and 6a–r). The qRT-PCR results showed that the expressions of DL, OsMADS1, and OsMADS6 were up-regulated, while the expression of G1 was down-regulated in the young panicles <5 cm in length (Figs. 5a, j and 6a and j).

Next, we further investigated the expression levels of these organ identity genes using in situ hybridization. In the wild-type spikelets, G1 was mainly expressed in the sterile lemmas at stages Sp4 to Sp8, and its transcriptional signals were also detected in the palea during the early stages of spikelet development (Fig. 5b–e). In contrast, G1 was expressed in the elongated rudimentary glumes and sterile lemmas of lrg1 (Fig. 5o–r). These results indicated that the elongated organs had a lemma identity.
In stages Sp4 to Sp8, OsMADS1 transcriptional signals were found in the lemma and palea of the wild-type spikelet (Fig. 6b–e); however, ectopic OsMADS1 expression was detected in the elongated rudimentary glumes and sterile lemmas of the lrg1 mutant (Fig. 6f–i). OsMADS6 transcripts were found in the floral meristem, palea margins, lodicule, and pistils in the spikelets of both the lrg1 mutant and the wild type at stages Sp4 to Sp8, but ectopic OsMADS6 signals were also detected in the receptacle and rachilla of the lrg1 spikelets (Fig. 6k–r). Taken together, these results show that LRG1 positively or negatively regulates the expressions of G1, DL, OsMADS1, and OsMADS6, and that its mutation causes their ectopic expression or absence of expression, affecting the formation of the rudimentary glumes and sterile lemmas.

Fig. 5. (Color online) Expression of G1 and DL genes in the wild type and lrg1 mutant floret. (a) qRT-PCR expression analysis of G1 in young panicles. (b)–(e) Expression of G1 gene in the wild-type floret. (f)–(i) Expression of G1 gene in the lrg1 mutant floret. (j) qRT-PCR expression analysis of DL in young panicles. (k)–(n) Expression of DL gene in the wild-type floret. (o)–(r) Expression of DL gene in the lrg1 mutant floret. esl, elongated sterile lemma; le, lemma; pa, palea; pi, pistil; 0.5P, 0.5 cm panicles; 0.5–2P, 0.5–2 cm panicles; 2–5P, 2–5 cm panicles. Bars = 50 μm. Error bars mean S.D. **P < 0.01.

3.10. LRG1 may act as a transcriptional repressor by interacting with the TPRs

The N-terminal and C-terminal regions of LRG1 each contain a typical EAR motif. Previous studies have shown that proteins containing EAR motifs can often interact with TPR1, TPR2, and TPR3 [42]. We used a yeast two-hybrid assay to verify whether LRG1 interacts with rice OsTPR1, OsTPR2, and OsTPR3. We constructed a vector containing the coding region of LRG1 in the GAL4 DNA-activating domain (AD), and transformed the coding regions of OsTPR1, OsTPR2, or OsTPR3 into the GAL4 DNA-binding domain (BD). AD-LRG1 was transformed into yeast competent cells alongside either BD-TPR1, BD-TPR2, BD-TPR3, or the pGBK7 vector.
The pGBK7-53 and pGADT7-T were also co-transformed into yeast competent cells as a positive control. The yeast cells co-transformed with AD-\textit{LRG1} and BD-\textit{OsTPR1}, AD-\textit{LRG1} and BD-\textit{OsTPR2}, AD-\textit{LRG1} and BD-\textit{OsTPR3}, or pGBK7-53 and pGADT7-T could survive in a culture medium lacking histidine, tryptophan, leucine, and adenine (SD/-Ade/-His/-Trp/-Leu), whereas yeast cells co-transformed with AD-\textit{LRG1} and pGBK7 could not grow normally (Fig. S7a online). We further analyzed the purified prokaryote-expressed protein using a GST pull-down assay. These results further confirmed that \textit{LRG1} can directly interact with \textit{OsTRP1}, \textit{OsTRP2}, and \textit{OsTRP3} (Fig. S7b online). These data suggest that \textit{LRG1}, a transcriptional inhibitor functioning in the nucleus, may perform its inhibitory role by interacting with \textit{OsTRP1}, \textit{OsTRP2}, and \textit{OsTRP3}.

4. Discussion and conclusion

4.1. \textit{LRG1} is an important regulator of grain size

Grain size is limited by grain length, grain width, and grain thickness [43]. So far, several genes affecting these traits have been reported, but the molecular mechanisms involved in regulating grain size have not yet been fully elucidated.
In this study, we cloned a novel regulatory factor, LRG1, involved in determining rice grain size. The mutation of LRG1 alters the grain size, with lrg1 grains being shorter but wider than those of the wild type. SEM revealed that the cell numbers and cell lengths were reduced along the longitudinal axis of the lrg1 mutant spikelets, while paraffin sections were used to reveal that the cell sizes in the lrg1 hull cross section were larger than those of the wild type.

Organ size is usually determined by cell proliferation and cell expansion. Several genes are known to affect grain size by affecting cell proliferation and cell expansion, including GS2, GS3, G5, GW2, GW8, BG1, BSG1 (BEAK-SHAPED GRAIN 1/TRIANGULAR HULL 1) [44], GFI1 (GRAIN INCOMPLETE FILLING 1) [45], G3, G7, and OsFBK12 [46]. The mutation of LRG1 affected the expression levels of GS2, GS3, G5, GW2, BG1, and OsFBK12. Our qRT-PCR results showed that the expression levels of the cell cycle-related and cell expansion-related genes in the lrg1 mutant were significantly different to their levels in the wild type. A further analysis using flow cytometry revealed that the cell proliferation rate in the lrg1 mutant during young panicle development was slower than that of the wild type, consistent with the decreased cell numbers along the longitudinal axis. These findings suggest that LRG1 affects cell proliferation and cell expansion by regulating the expression levels of these genes, thereby regulating grain size.

4.2. LRG1 affects the determinacy of the spikelet meristem

About 38% of lrg1 spikelets produced an extra lemma-like organ, the identity of which was confirmed using both morphological and molecular evidence. The lrg1 spikelets therefore formed a terminal floret and a secondary floret bearing only one lemma. Similarly, in the mfs1 and tob1 (tongari-boushi) mutants, some spikelets developed an extra lemma-like organ between the lemma and the sterile lemma [18,47], while in the snb mutant, some spikelets produced several rudimentary glumes, extra lemma-like organs, and even extra florets with floral organs [16,17]. In the df1/eg1 mutant, some spikelets produced an extra lemma-like organ or two complete florets [13]. Recently, LFI1 (LATERAL FLORET 1) and FON4 were also reported to regulate spikelet meristem determinacy [19,48]. In the fl1 mutant, one or two lateral florets were formed in the axilla of the sterile lemma, while in the fon4 mutant, some spikelets formed extra lemma-like organs while other spikelets developed two lemma-like and palea-like organs and four lodicules, suggesting the formation of two florets. These results revealed that LRG1, MFS1, TOB1, SNB, DF1/EG1, LFI1, and FON4 regulated the transition from the spikelet meristem to the floral meristem, and that their mutation resulted in the formation of extra florets in the spikelets, indicating the alteration of spikelet meristem determinacy. This loss of spikelet determinacy occurred before the presence of the rudimentary glumes in the snb mutant, but it occurred after the formation of the sterile lemma in the lrg1 and mfs1 mutants, suggesting that LRG1 and MFS1 function later in development than SNB.

4.3. LRG1 regulates sterile lemma and rudimentary glume development

In our study, the lrg1 mutant formed elongated sterile lemmas and rudimentary glumes, which were transformed into lemma-like organs with a lemma identity. Similarly, in the pap2/omsads34 and asp1 (aberrant spikelet and panicle 1) mutants, the rudimentary glumes and sterile lemmas were elongated and exhibited a lemma-like identity [10–12,49], and in the g1, eg1, and fon4 mutants, the sterile lemmas were homeotically transformed into lemmas [9,13,19]. These findings indicate that LRG1, PAP2/OMSADS34, ASP1, G1, EG1, and FON4 affect the identities of the sterile lemmas and rudimentary glumes by inhibiting the homologous transformation of the rudimentary glumes and sterile lemmas into lemmas.

The rice spikelet consists of a terminal fertile floret with two sterile lemmas and two rudimentary glumes. Two prevailing hypotheses about the origin and evolution of the sterile lemma have been proposed [50]. One hypothesis is that the ancestor of Oryza produced three-floret spikelets consisting of a terminal floret and two lateral florets. The two lateral florets gradually degenerated during evolution, and are now represented by the two sterile lemmas of the rice spikelet [9,51]. The other hypothesis is that the ancestor of Oryza only produced one floret, and the sterile lemmas and rudimentary glumes should be considered to be degenerate bract-like organs [52]. In the lrg1, pap2/omsads34, asp1, g1, eg1, and fon4 mutants, the sterile lemmas were homeotically converted into lemmas. In the fl1 mutant, the lateral florets were induced between the sterile lemmas and the terminal floret. These results strongly support the first hypothesis [48]. However, the lrg1, pap2/omsads34, and asp1 mutants also caused the homeotic transformation of the rudimentary glumes into sterile lemmas or lemmas. In addition, the mfs1, fjp, snb, and ids1 mutants developed rudimentary glume-like sterile lemmas, suggesting that the degraded sterile lemmas possess rudimentary glume identities [14,16–18]. These data reveal that the rudimentary glume, the sterile lemma, and the lemma are homologous organs.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

Qian Qian, Deyong Ren and Yuchun Rao designed the research. Qiankun Xu, Haiping Yu, SaiSai Xia, Yuanjiang Cui, Xiaoqi Yu, Lan Shen, Qiang Zhang, Dali Zeng, Zhenyu Gao, Jiang Hu, Guangheng Zhang, Li Zhu, Longbiao Guo, Deyong Ren performed research. Qian Qian, Deyong Ren and Qiankun Xu wrote the article. Qian Qian and Deyong Ren agrees to serve as the authors responsible for contact and ensures communication.

Appendix A. Supplementary materials

Supplementary materials to this article can be found online at https://doi.org/10.1016/j.scib.2020.01.019.

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