Cloning of *Long Sterile Lemma (lsl2)*, A Single Recessive Gene Regulating Spike Germination in Rice (*Oryza Sativa L.*)

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Abstract

Background: Rice is a typical monocotyledonous plant and an important cereal crop. The structural units of rice flowers are spikelets and florets. Floral organ development and spike germination affect rice reproduction and yield.

Results: In this study, we identified a novel *long sterile lemma* (*lsl2*) mutant from an EMS population. First, we mapped the *lsl2* gene between the markers Indel7-22 and Indel7-27, which encompasses a region of 25 kb. The rice genome annotation indicates the presence of four candidate genes in this region. Through gene prediction and cDNA sequencing, we confirmed that the target gene in the *lsl2* mutant is allelic to *LONG STERILE LEMMA1* (*G1*)*/ELONGATED EMPTY GLUME* (*ELE*), hereafter referred to as *lsl2*. Further analysis showed a one-amino acid change, serine (Ser) 79 mutated to proline (Pro), in the lsl2 protein had compared with LSL2, which may change the function of the LSL2 protein. The *knockout experiments* showed that the *lsl2* gene is responsible for the long sterile lemma phenotype. The *lsl2* gene may reduce the damage caused by spike germination by decreasing the seed germination rate, and yet other agronomic traits of rice are not affected in the *lsl2* mutant. Taken together, our results demonstrate that the *lsl2* gene will have specific application prospects in future rice breeding.

Conclusions: The *lsl2* gene is responsible for the long sterile lemma phenotype, and may reduce the damage caused by spike germination by decreasing the seed germination rate.

Background

The flower forms of angiosperms are diverse. Flower morphology is the result of interactions of an established genetic programme, physical forces, and external forces induced by the pollination system [1]. To establish this diversity, it is essential to identify the floral organs and control the fate of meristems. In eudicots, flowers are generally composed, from outer whorls to inner whorls, of sepals (whorls), petals (whorls), stamens (whorls), and pistils (whorls). Based on molecular and genetic analyses in several eudicot species, including *Arabidopsis thaliana*, snapdragon (*Antirrhinum majus*), and petunia (*Petunia hybrida*), an ABC model has been proposed that determines the characteristics of each organ and control floral meristem determinacy by combining A/B/C/D gene groups [2–7]. According to the model, three homologous genes control the formation of flower organs. A-functional genes independently specify sepal formation; the combination of A- and B-functional genes determine petal identity; B- and C-functional genes jointly regulate stamen development; and only the C-functional gene specifies the innermost carpels. This genetic model applies not only to eudicots but also to monocots, including some grass species such as rice (*Oryza sativa L.*) and maize (*Zea mays*) [8–12].

Rice is a typical monocotyledonous plant and an important cereal crop. The structural units of rice flowers are spikelets and florets. The spikelet is the main unit of the rice inflorescence and contains a fertile floret and a pair of empty glumes (also known as "a sterile lemma") [13]. The floret consists of a lemma, two lodicules (equivalent to petals), six stamens, and a pistil [14–15].

A previous study showed that *Sepallata* (*SEP*) subfamily members and the *LOFSEP* subgroup of *MADS*-box genes play an important role in the development of rice flowers. During flower development, two *SEP3* homologues and *OsMADS7/8* are expressed in the inner three whorls and show redundant functions [16]. In addition to *OsMADS7* and *OsMADS8*, *LEAFY HULL STERILE1* (*OsLHS1*), *OsMADS5* and *OsMADS34/PAP2* have been reported to function in flower development [17]. Some early studies reported that *OsMADS34/PAP2* not only regulates spikelet meristem identity and ovule development but also empty glume development. In *Osmads34/pap2* mutants, empty glumes are elongated to form leaf-like or lemma-like organs [17–19]. The results of evolution and sequence analysis of *OsMADS34/PAP2* support the hypothesis that the empty glumes of rice originate from the degenerated floret lemma, named the rudimentary lemma [19]. *LONG STERILE LEMMA1 (G1)/ELONGATED EMPTY GLUME (ELE)* encodes a DUF640-containing protein that determines the identity of the empty glumes. When *G1/ELE* is mutated, the empty glumes become lemma-like organs [20–21]. Interestingly, natural mutations in the empty glumes cause similar homeotic conversions in the genome of allotetraploid *Oryza grandiglumis*, suggesting that empty glumes may constitute a series of lemma homologues modified by *G1/ELE* [20].

Although the molecular mechanisms that control the development of reproductive organs in rice are well known, the role of the long sterile lemma and whether it will affect the agronomic character of rice remain unclear. In this study, *long sterile lemma 2* (*lsl2*), a new strong mutant allele of *G1*, was identified in the ZH11 background. We mapped *lsl2*, analysed the 3-D structure of the LSL2 protein, and found that the lsl2 protein contains a one-amino acid change of serine (Ser) 79 to proline (Pro), which is predicted to alter the structure of the LSL2 protein. We also report the molecular cloning of *lsl2* and agronomic character analysis of the *lsl2* mutant. Together, the results indicate that *lsl2* has specific value in rice crossbreeding.

Methods

Plant materials

Indica rice CO39 and *japonica* ZH11 were provided by tPlant Immunity Center, Fujian Agriculture and Forestry University and were preserved at the Rice Research Institute, Fujian Academy of Agricultural Sciences, China. The long sterile lemma mutant in the background of ZH11 was screened by the M_2 population treated with ethyl methanesulfonate (EMS) and named *long sterile lemma 2* (*lsl2)*. Approximately 800 plants in the M_1 population and 6000 plants in the M_2 population were field-grown at Fuzhou Experimental Station in Fujian Academy of Agricultural Sciences in 2016 and 2017, respectively.

In the summer of 2018, the *lsl2* mutant was hybridized with the rice cultivars CO39 and ZH11 as pollen donors. The F_1 seeds were sown at Sanya (18.14 northern latitude, 109.31 east longitude) Experimental Station in Hainan Province in the spring, and F_2 seeds were harvested. The F_2 seeds *lsl2* and ZH11 were planted at Fuzhou (26.08 northern latitude, 119.28 east longitude) Experimental Station in Fujian Province in the summer of 2019. Plant height, panicle number per plant, flag leaf length and width,

spikelet number per panicle, and seed setting rate were measured at maturity. The segregation ratios of mutants versus wild-type were examined after maturity.

All plants were planted in accordance with standard commercial procedures, with spacing between rows of 13.3 cm and between rows of 26.4 cm, and field management generally followed normal agricultural practices.

Construction of the mapping population

The *lsl2* mutant (*japonica*) was hybridized with CO39 (*indica*) to produce a mapping population. The F_2 population was constructed through self-crossing of the F_1 population, and 1084 mutant-phenotype plants in the F_2 population were selected for fine mapping.

Microsatellite analysis

Simple sequence repeat (SSR) primers were obtained from the published rice database (http://www.Gramene.org/microsat/ssr.htm1). Indel markers were designed by manually comparing the genome sequences between *japonica* (cv. Nipponpare) [22] and *indica* (cv. 93-11) [23]. First, the bacterial artificial chromosome (BAC) clone sequences of *japonica* and *indica* were compared, and then Primer premier 5.0 was used to design primers for polymorphic regions between the two rice subspecies, which were used for gene localization.

PCR (Polymerase chain reaction) amplification and marker detection

The CTAB method [24] was used to extract plant DNA from frozen leaves of rice plants, with minor modifications. For PCR amplification, every 20-μL reaction mixture contained 30 ng DNA, 0.4 μM of each primer, and 2× Es Tag MasterMix (Dye). The amplification procedure was performed with the following programme: 2 min at 94°C, 33 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, with a final extension of 2 min at 72°C. The PCR products were electrophoresed in 3% agarose gels with ethidium bromide staining [25].

Bulked segregant analysis

Bulk segregant analysis (BSA) was applied to identify markers associated with target genes. DNA from the leaves of 15 randomly selected mutant plants of the F_2 population was used to construct a mutant DNA library. Linkage was detected by SSR markers distributed in the rice genome, with DNA extracted from the *lsl2* mutant and CO39 used as a control. The bands of markers linked to the mutant genes were the same as those of the *lsl2* mutant.

Molecular mapping of the *lsl2* gene

The band types of mutants (*lsl2 lsl2*) and (*LSL2LSL2*) were denoted as 1 and 3, respectively; 2 was used to represent a heterozygote (*lsl2 LSL2*). Linkage analysis between the *lsl2* locus and the SSR markers

was performed using MAPMAKER version 3.0 software [26]. Map distances were estimated using MapDraw V2.1 [27]. The linkage map in this study was basically the same as that reported previously [28].

First, 326 SSR markers were selected from the rice molecular map for polymorphism investigation between *lsl2* and CO39 [29]. Among them, 205 pairs showed polymorphism. According to these 205 markers, 15 mutant strains and 15 normal strains were selected from the F_2 population for linkage analysis of the *lsl2* locus. Second, to delineate the gene to a smaller region, we identified 1084 mutants from the F_2 population of *lsl2* × CO39, and Indel markers from the open rice genome sequences were designed to predict the likelihood of polymorphisms between *lsl2* and CO39 by comparing sequences from *Nipponbare* (http://rgp.dna.affrc.go.jp/) and the *indica* cultivar 93-11 (http://rice.genomics.org.cn/).

Physical map construction

Bioinformatics analysis was performed using BAC and P1-derived artificial chromosome (PAC) clones of cv. Nipponbare released by the International Rice Genome Sequencing project (IRGSP, http://rgp.dna.affrc.go.jp/IRGSP/index.html) to construct a physical map of the target gene. The clones were anchored to the target gene binding markers, and sequence alignment was performed using pairwise BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/b12.html).

Bioinformatics correlation analysis

Candidate genes were predicted according to the existing sequence annotation database (http://rice.plantbiology.msu.edu/; http://www.tigr.org/). The DNA and amino acid sequences of *lsl2* and *LSL2* were used for a complete alignment with Clustal X version 1.81. The 3-D structures of the lsl2 and LSL2 proteins were predicted and analysed (https://swissmodel.expasy.org/). Haplotype analysis of *lsl2* and *LSL2* was also performed (http://www.rmbreeding.cn/Genotype/haplotype).

Targeted mutagenesis of *LSL2* in rice with CRISPR/Cas9

The *LSL2* gene in ZH11 was targeted with one gRNA spacer that spanned 106 bp of the first exon of the gene. gRNA spacer sequences with high specificity (Supplementary Table 1) were designed using the CRISPR-plant database and website [30], and genome-editing mutations of the target gene in regenerated plants were evaluated. Chromosomal deletions and insertions were detected by PCR using primers located in gene target sites. PCR products were selected from the transgenic CRISPR-edited lines for sequencing to identify specific mutations. Double peaks were resolved with the degenerate sequence decoding method [31]. Primers for the CRISPR/Cas9 study are listed in Supplementary Table 1.

Measurement of germination rates

Each rice material was incubated in a plant-light incubator for 24 hours; 100 seeds for each material were germinated, which was repeated 4 times for each material. The germination test was conducted according to the standard germination test method. The germination bed consisted of paper, and the test

was carried out at 25°C. The number of seeds germinating was recorded after 2 days, and the germination rate was continuously recorded until the 7th day. Moisture and temperature conditions were maintained.

Results

Main agronomic characteristics of *lsl2*

To elucidate the genes that regulate flower development in rice, we screened for a floret mutant phenotype among an EMS-mutagenized population and identified a *long sterile lemma 2* (*lsl2)* mutant in the ZH11 background. Phenotypic comparisons between the *lsl2* mutant and wild-type ZH11 are presented in Table 1. The results showed no significant differences in major agronomic traits, including plant height, panicle length, number of effective panicles, spikelets per panicle, seed-setting rate, 1,000-grain weight, grain length and grain width.

Phenotypic observation and analysis of the *lsl2* mutant

In the vegetative stage, ZH11 and *lsl2* plants showed indistinguishable phenotypes, but their spikelets displayed different phenotypes from the boot stage to the mature stage (Table 1, Fig. 1a, b). The *ls12* mutants exhibited a much longer sterile lemma than that of ZH11, though other components of the spikelet were the same (Fig. 1a, b). Interestingly, there was no significant difference in grain size or brown rice size between *lsl2* and ZH11 after maturation (Table 1, Fig. 1c, d).

We compared the germination rates of seeds between *lsl2* and ZH11 and found that on the second day, wild-type ZH11 started sprouting (69.3%) but that the *lsl2* mutant had barely begun to germinate (2.3%) (Fig. 2 and Table 2). Compared to wild-type, the *lsl2* mutants showed obviously reduced germination rates from the second day to the fourth day (Table 2).

Genetic analysis of the *lsl2*mutant

To determine whether the *lsl2* mutant is caused by a single gene, we next crossed the *lsl2* mutant with ZH11. The F_1 generation showed normal phenotypes, and the F_2 population showed Mendelian segregation (Table 3). Indeed, segregation between the wild-type and mutant plants fit a 3:1 segregation ratio in the two F_2 populations (χ^2=0.124–0.462, P>0.5), indicating that the *lsl2* mutant phenotype is controlled by a single recessive gene.

Initial localization of the *lsl2* gene

To determine which gene mutation causes the *lsl2* phenotype, we next mapped the *lsl2* gene. Two SSR markers, RM4584 and RM2006, located on rice chromosome 7, were found to be associated with mutant traits in 193 F_2 individuals. Based on the recombination frequency, the genetic distance between RM4584 and RM2006 was calculated to be 28.8 cM. Therefore, *lsl2* is located in a 28.8-cM region on chromosome 7 flanked by SSR markers RM4584 and RM2006 (Fig. 3a).

Fine mapping of the *lsl2* gene

To delineate the gene to a smaller region, an accurate map was constructed between RM4584 and RM2006 by using published markers (Table 4). Through genetic linkage analysis, the *lsl2* gene was mapped between the molecular markers RM8059 and RM427, with a distance of 7.6 cM (Fig. 3b). For further mapping, genotyping of all recombinant genes was performed using 9 polymorphic markers (Table 4). The results showed that the *lsl2* gene is located between the molecular markers Indle7-13 and Indle7-15, with a physical distance of 205 kb (Fig. 3c and Table 4). To fine map the *lsl2* gene, seven polymorphic Indel markers for recombinant screening (Table 4) detected one, one, three, three, six, seven and eleven recombinant plants, respectively (Fig. 3d). Thus, we precisely localized the *lsl2* gene between the molecular markers Indel7-22 and Indel7-27, with a physical distance of 25.0 kb.

Candidate genes in the 25.0-kb region

Four candidate genes are annotated (LOC_Os07g04660, LOC_Os07g04670, LOC_Os07g04690, LOC_Os07g04700) in this 25.0-kb region (Fig. 3e). According to the available annotation database, these four genes all have a corresponding full-length cDNA. LOC_Os07g04660 encodes white-brown complex homologue protein 16, LOC_Os07g04670 a DUF640 domain containing protein, LOC_Os07g04690 UDP-arabinose 4-epimerase 1 and LOC_Os07g04700 an MYB family transcription factor.

Sequence analyses of the *lsl2* gene

To analyse which gene causes the mutant phenotype, we sequenced the above four genes in ZH11 and *lsl2* and found only a single 1-bp change (T to C) in LOC_Os07g04670 between wild-type ZH11 and the *lsl2* mutant. No other differences in the remaining three gene sequences were observed. Thus, we speculated that the LOC_Os07g04670 locus corresponds to *lsl2*. Interestingly, the *G1/ELE* gene, encoding a DUF640 domain-containing protein, is present in this locus [20]. Based on phenotypic similarity and localization analysis, we hypothesized that the long sterile lemma phenotype of *lsl2* may be caused by functional changes in the product of the LOC_Os07g04670 locus. These results suggest that the *lsl2* gene may be allelic with *G1/ELE*.

Analysis of the open reading fragment (ORF) showed one exon and no intron for the *LSL2* gene (LOC_Os07g04670). *lsl2* is a 1-bp mutant that results in the exchange of a serine (Ser) for a proline (Pro) (Fig. 4). Ser is a polar amino acid, whereas Pro is nonpolar. Such a mutation may alter the function of a protein.

The *lsl2* geneis responsible for the long sterile lemma phenotype

To confirm that the mutation phenotype can be attributed to *lsl2*, we examined whether knockout of *LSL2* in the cultivar ZH11 would lead to the long sterile lemma phenotype. One sequence-specific guide RNA (sgRNA) was designed to knock out the *LSL2* gene by using the CRISPR/Cas9 gene editing system. A total of three plants from three independent events were obtained and confirmed by sequencing to carry insertions and deletions in the target sites (Table 5).

We then investigated the panicle characteristics of these three homozygous lines after maturity and found that all three exhibit a long sterile lemma phenotype (Fig. 5), indicating that knockout of *LSL2* in ZH11 leads to the long sterile lemma mutation phenotype.

Analyses of 3-D structures between the LSL2 protein and the lsl2 protein

By further simulating the 3-D structure of the protein, we found changes between the lsl2 protein and the LSL2 protein (Figure 5). Moreover, we observed a significant change in protein structure when residue 79 of LSL2 was changed from Ser to Pro (Fig. 6).

Haplotype analysis of the *LSL2* gene

To further investigate the genetic and evolutionary characteristics of the *LSL2* gene, we performed SNP calling and haplotype analysis of the 3,000 sequenced rice genomes available in the CNCGB and CAAS databases [32] and found 492 haplotypes for the *LSL2* gene, with 49 haplotypes among more than 15 rice resource materials (Supplementary Table 2). However, in the 3,000 sequenced rice genomes, no haplotype or SNP was found for the *lsl2* mutant.

Discussion

The mechanism for controlling the development of empty glumes and lemmas

The molecular mechanism that determines the development of the lemma differs from that of the empty glume [13]. Through the analysis of *Osmads34*/*pap2* mutant plants Lin et al proposed that the empty glume originates from the lemma and named it the basic lemma [19]. Yoshida et al identified glumes as remnants of two lower reducing florets and named them the sterile lemma [20].

Further research and molecular evidence of lemma development will provide clues for determining the identity of empty glumes. Further investigations are also necessary to reveal the key genes that play a role in the lemma identification of glumes.

Genetic and evolutionary analyses of the *LSL2* gene

Haplotype analysis of the 3,000 sequenced rice genomes showed 492 haplotypes for the *LSL2* gene (Supplementary Table 2). However, no haplotype or SNP for the *lsl2* mutant, which contains a T to C transition, was found in the 3,000 sequenced rice genomes. We speculate that mutation at this site would be strongly selected against in natural selection and only be the result of manual selection. For example, the phenotypes of *lsl2* may be inconsistent with the expectations of the breeders; therefore, it was gradually eliminated by manual selection.

As the 3-D structures of LSL2 and lsl2 based on the simulation showed that this amino acid change alters the protein structure (Fig. 6), we speculate that this change may affect the specific function of LSL2, such as binding activity to its target protein.

Analysis of the application prospect of the*LSL2* gene

Although the *lsl2* mutation did not affect major agronomic traits, it remains unclear whether it affects the internal characteristics of rice. By comparing the germination rates of seeds between *lsl2* and ZH11, we observed obviously reduced germination rates from the second day to the fourth day in the *lsl2* mutant (Table 2). We propose that the most likely reason is that the longer sterile lemma of *lsl2* may inhibit the growth of embryos.

Spike germination in rice is closely related to the seed germination rate. In the production of hybrid rice worldwide, spike germination is a prominent issue that affects both the yield and processing quality of rice, thereby causing economic losses of different degrees [33]. In this study, we observed that the *lsl2* mutation reduced the damage caused by spike germination by decreasing the seed germination rate. Interestingly, other agronomic traits of rice were not affected in the *lsl2* mutant (Table 1). Therefore, the *lsl2* gene has specific application prospects in rice breeding. First, breeders can develop excellent conventional rice varieties using *lsl2*. Second, the *lsl2* gene is controlled by a single recessive gene (Table 3); thus, to breed a new hybrid rice variety, breeders can transfer this gene into both restorer and sterile lines using molecular marker assistance.

Conclusions

In this study, we identify a novel *long sterile lemma* (*lsl2*) mutant from an EMS population. The *lsl2* gene is responsible for the long sterile lemma phenotype, and a one-amino acid change, serine (Ser) 79 mutated to proline (Pro), in the lsl2 protein has compared with LSL2, which may change the function of the LSL2 protein. The results of this study indicate that the *lsl2* mutant may reduce the damage caused by spike germination by decreasing the seed germination rate.

Declarations

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Not applicable.

Authors' contributions

DY planned and carried out the experiments and data collection and wrote the manuscript with input from all authors. NH, XZ, YZ, ZX, CC and FH were involved in conducting the experiments, data collection and analyses. All authors discussed the results and contributed to the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1
Comparison of the main agronomic traits between ZH11 and *lsl2*

Traits	**ZH11**	***lsl2***
Plant height (cm)	77.62 ± 1.86	78.12 ± 1.82
Panicle length (cm)	25.22 ± 1.22	25.46 ± 1.20
Number of effective panicle	8.64 ± 1.04	8.84 ± 1.08
Spikelets per panicle	128.46 ± 4.26	132.36 ± 3.84
Seed-setting rate (%)	96.52 ± 0.16	97.38 ± 0.20
1,000-grain weight (g)	25.02	25.44
Grain length (mm)	8.57 ± 0.12	8.65 ± 0.13
Grain width (mm)	2.49 ± 0.08	2.52 ± 0.04
Brown rice length (mm)	5.72 ± 0.10	5.68 ± 0.08
Brown rice width (mm)	2.12 ± 0.04	2.14 ± 0.03

* Difference between ZH11 and *lsl2* at $P < 0.05$; **at $P < 0.01$. Data are derived from the trial performed at Fuzhou experimental station in October 2019.

Table 2
Comparison of germination rates between ZH11 and *lsl2*

Number of days / **Name of the material**	**1d**	**2d****	**3d****	**4d ***	**5d**	**6d**	**7d**
Germination rate of ZH11 (%)	0	69.3	94.8	95.3	95.8	96.8	96.8
Germination rate of *lsl*2 (%)	0	2.3	63.5	82.5	94.6	95.6	95.6

* Difference between ZH11 and *lsl2* at $P < 0.05$; **at $P < 0.01$

Table 3
Segregations of F_2 populations produced from crossing of the *lsl2* mutant

Crosses	**F_1 phenotype**	**F_2 population**			**χ^2(3:1)**	**P**
		Wild-type plants	**Mutant plants**	**Total plants**		
lsl2/ZH11	Normal type	180	57	237	0.462*	0.5–0.75
ZH11/*lsl2*	Normal type	198	64	262	0.124*	> 0.9

* Denotes the segregation ratio of normal plants to mutant plants complying with 3:1 at the 0.05 significance level.

Table 4
Indel and SSR molecular markers used for fine mapping of the *lsl2* gene

Marker	Sequence of forward primer	Sequence of reverse primer
RM8059	GGAAAGACCAGTTTAGAGCAATGG	AGCTAGATCCCTTGTTTCACACG
RM427	TCACTAGCTCTGCCCTGACC	TGATGAGAGTTGGTTGCGAG
RM4098	CGTTTGGATGAAGAAGAAGA	AGTGTTCGTTTCGGATTAGA
Indle7-2	CAGATATGATGTTCTTGCCCTTGC	GCTTGCCAGATCACCTACCTACC
Indle7-3	CGGAGCTGTTGCCGTTCTGC	CGATGTGCCATGTCAGGATGACC
Indle7-5	CCTACCGCGTCATTCACATGC	GACAAGATCGACAGCCGCTACG
Indle7-6	TCACTCACACACTGACTGACACG	TCTCGTCGGAGAAGAAGATGAGC
Indle7-9	CACTATGGATCTTGGTGGTCAAGG	TGCTATCTGCTACCGTCAACACG
Indle7-13	GTAGGACATGAAGGCGGCTAGG	ATCTCCTGCCACTGCACACC
Indle7-15	CGTCCATATCAAACCTCTTCTTCC	GTAACATTCCCTCCCGAACTCC
Indle7-16	GGTGCAGACTACCTAAATATGACG	GTAAACCGATGGCTTAGAGTCC
Indle7-19	AACGGGAGATCACAGGAATTTGC	GTGTTCGACTCGTCTCCATTTCG
Indle7-22	ACAGTGAAAGCCACTACCAT	CTTGACTGGGTGTCCATATT
Indle7-27	TAGGTGCAACTTCTTGAAGTG	GATCCCCTGTTCATTTGTAATT
Indle7-30	AGGGGCGCACAGCGGGGAGGGTC	TCAATCCACGGAATCCACGAC
Indle7-35	GATTTCAGAAGATGTTTGGG	GGTTTCCCAGTTTCTGTCTT
Indle7-38	TGATTTTATCCTCGTCTTCC	AACATGCGCATATGTAACTG
Indle7-40	TCTCTTCTCTCTTGCTTCTC	ATGTCAATTTGATGGGATGT
Indle7-42	TGGAAAAGAACTTCAATGCT	TTGAATCACCACAATTTAGC

Table 5. Mutation site of three targeted mutant lines

Line	Target type	Mutation site
Line1	gRNAs	ACTGGCAGACCTTCACGCAGTTACCTCGCCGCGCACCGCCCGC (Insert 1bp)
Line2	gRNAs	ACTGGCAGACCTTCACGCAGT--CTCGCCGCGCACCGCCCGC (deletion 2bp)
Line3	gRNAs	ACTGGCAGACCTTCACGCAGT-CCTCGCCGCGCACCGCCCGC (deletion 1bp)

Figures

Figure 1

Phenotypes of ZH11 and the ls2 mutant. a: Grain phenotypes of ZH11; b: grain phenotypes of the ls2 mutant; c: brown rice phenotypes of ZH11; d: brown rice phenotypes of the ls2 mutant. Other than the much longer sterile lemma than ZH11, the lsl2 mutant displays no significant difference in grain size or shape compared to ZH11.

Figure 2

Comparison of germination rates between lsl2 mutant and ZH11 seeds. ZH11 showed a higher germination rate than the lsl2 mutant from the second day.

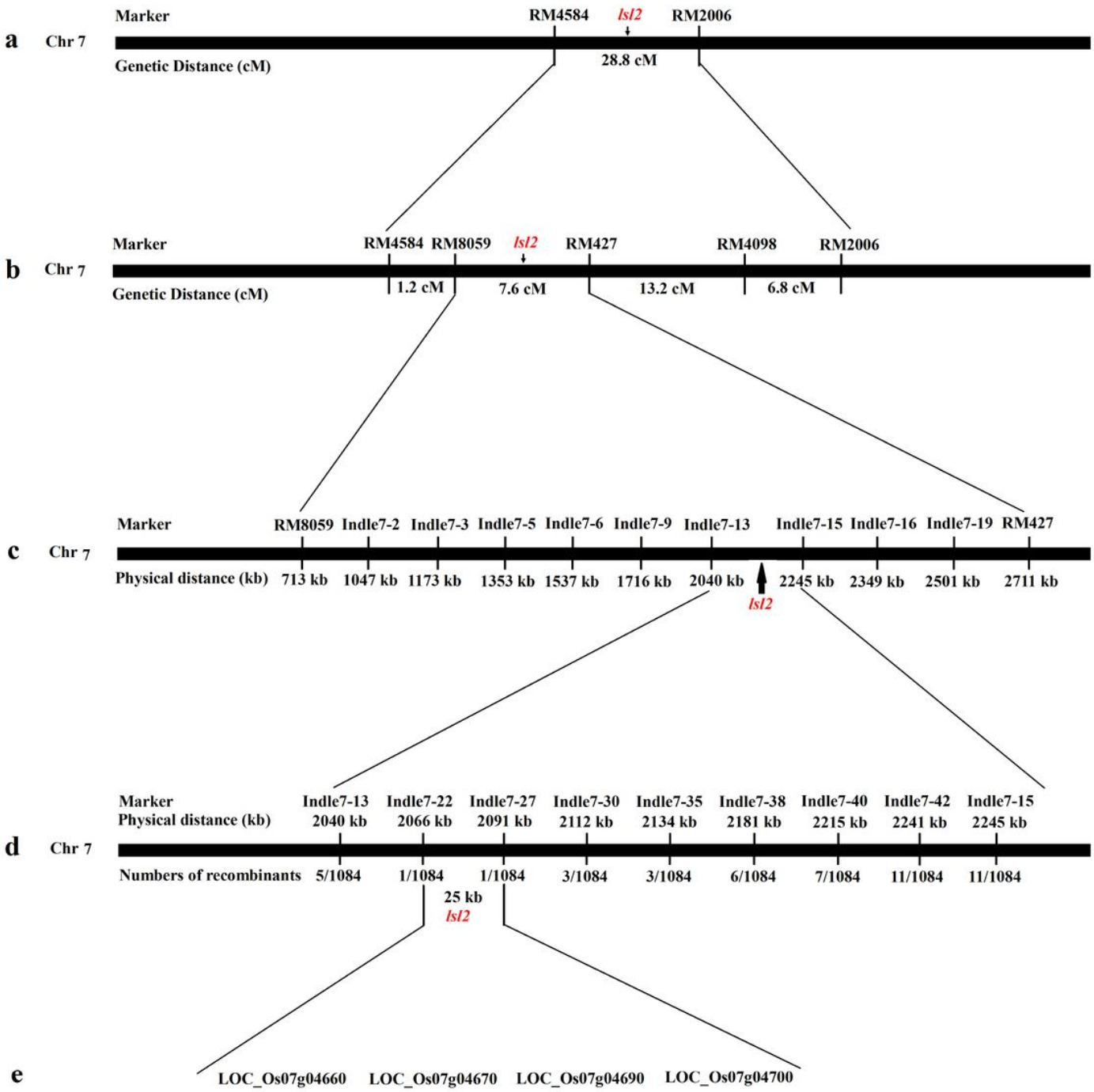

Figure 3

Genetic and physical maps of the lsl2 gene. a: Primary mapping of the lsl2 gene. The gene was mapped to the region between markers RM4584 and RM2006. b: Further mapping of the lsl2 gene. The gene was mapped to the region between markers RM8059 and RM427. c: Fine mapping of the lsl2 gene. The gene was mapped to the region between markers Indel7-13 and Indel7-15. d: High-resolution mapping of lsl2. The lsl2 gene was localized to a 25.0-kb region between markers Indel7-22 and Indel7-27, and the

recombinant number between markers and target gene is indicated under the linkage map. e: Candidate genes in the 25.0-kb target region.

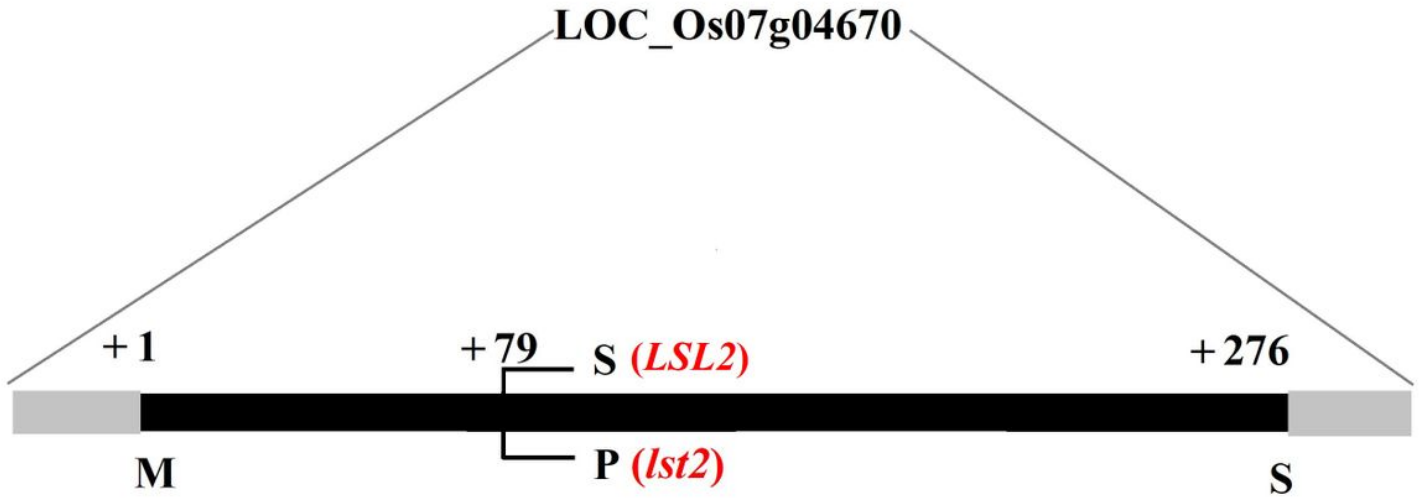

Figure 4

Structure comparison between the LSL2 protein and the lsl2 protein. There was only one amino acid substitution (S79P) between LSL2 and lsl2.

Figure 5

LSL2-knockout mutants show a long sterile lemma phenotype. The three knockout lines generated by CRISPR/Cas9 all exhibit a long sterile lemma phenotype.

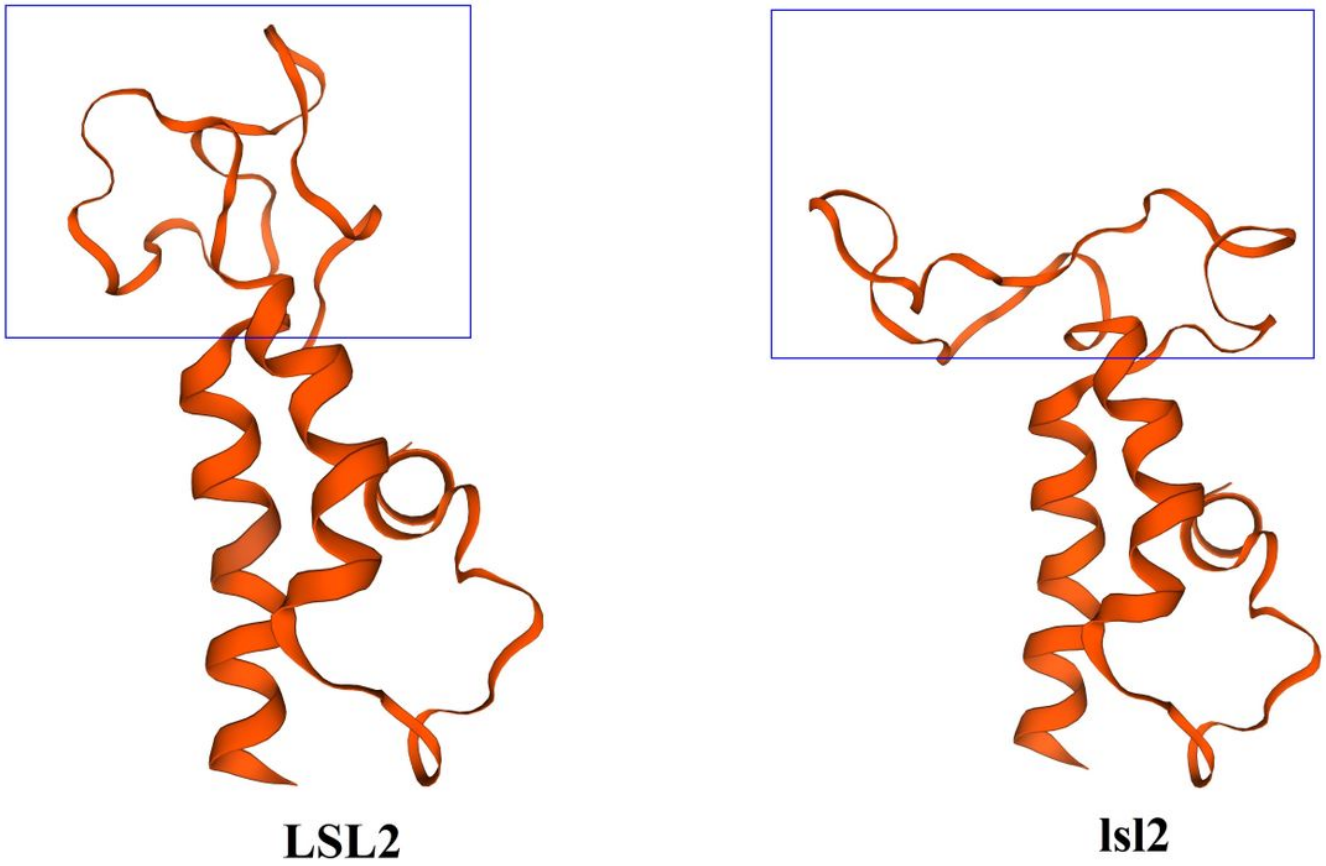

Figure 6

3-D structures of the LSL2 protein and the lsl2 protein. There are significant structural changes based on the Swiss-Model, and the 79th residue of the LSL2 protein is changed from a serine (S) to proline (P), which significantly changes the structure. The blue square indicates the site of the change in the 79th amino acid.

Supplementary Files

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- SupplementaryTables.docx