

# Cloning of *long sterile lemma (Isl2)*, a single recessive gene that regulates spike germination in rice (*Oryza sativa L.*)

dewei yang (✉ [dewei-y@163.com](mailto:dewei-y@163.com))

Fujian Academy of Agricultural Sciences

**Niqing He**

Fujian Academy of Agricultural Sciences

**Xianghua Zheng**

Fujian Academy of Agricultural Sciences

**Yanmei Zhen**

Fujian Academy of Agricultural Sciences

**Zhenxin Xie**

Fujian Academy of Agricultural Sciences

**Chaoping Cheng**

Fujian Academy of Agricultural Sciences

**Fenghuang Huang**

Fujian Academy of Agricultural Sciences

---

## Research article

**Keywords:** Rice (*Oryza sativa L.*), long sterile lemma mutant, molecular marker, gene cloning, application prospect, spike germination

**Posted Date:** October 13th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-50190/v2>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published at BMC Plant Biology on December 11th, 2020. See the published version at <https://doi.org/10.1186/s12870-020-02776-8>.

# Abstract

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

**Results:** In this study, we identified a novel *long sterile lemma* (*Isl2*) mutant from an EMS population. First, we mapped the *Isl2* gene between the markers Indel7-22 and Indel7-27, which encompasses a 25-kb region. The rice genome annotation indicated the presence of four candidate genes in this region. Through gene prediction and cDNA sequencing, we confirmed that the target gene in the *Isl2* mutant is allelic to *LONG STERILE LEMMA1 (G1)/ELONGATED EMPTY GLUME (ELE)*, hereafter referred to as *Isl2*. Further analysis of the *Isl2* and *LSL2* proteins showed a one-amino-acid change, namely, the mutation of serine (Ser) 79 to proline (Pro) in *Isl2* compared with *LSL2*, and this mutation might change the function of the protein. *Knockout experiments* showed that the *Isl2* gene is responsible for the long sterile lemma phenotype. The *Isl2* gene might reduce the damage induced by spike germination by decreasing the seed germination rate, but other agronomic traits of rice were not changed in the *Isl2* mutant. Taken together, our results demonstrate that the *Isl2* gene will have specific application prospects in future rice breeding.

**Conclusions:** The *Isl2* gene is responsible for the long sterile lemma phenotype and might reduce the damage induced by spike germination by decreasing the seed germination rate.

## Background

The flower forms of angiosperms are diverse, and flower morphology is the result of interactions among an established genetic programme, physical forces, and external forces induced by the pollination system [1]. Identifying floral organs and controlling the fate of meristems are essential for establishing this diversity. In eudicots, flowers are generally composed (from the outer to inner whorls) of sepals (whorls), petals (whorls), stamens (whorls), and pistils (whorls). Based on molecular and genetic analyses of several eudicot species, including *Arabidopsis thaliana*, snapdragon (*Antirrhinum majus*), and petunia (*Petunia hybrida*), an ABC model that determines the characteristics of each organ and controls floral meristem determinacy based on the combination of A/B/C/D gene groups has been proposed [2-7]. According to the model, three homologous genes control the formation of flower organs. A-function genes independently specify sepal formation; the combination of A- and B-function genes determines petal identity; B- and C-function genes jointly regulate stamen development; and only the C-function gene specifies the innermost carpels. This genetic model applies to not only eudicots but also 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, and spikelets and florets are the structural units of rice flowers. The spikelet is the main unit of the rice inflorescence and contains a fertile floret and a pair of sterile lemmas (also known as “a sterile lemma”) [13], and 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 have redundant functions [16]. In addition to *OsMADS7* and *OsMADS8*, *LEAFY HULL STERILE1* (*OsLHS1*), *OsMADS5* and *OsMADS34/PAP2* reportedly function in flower development [17]. Some early studies found that *OsMADS34/PAP2* regulates the identity of the spikelet meristem as well as ovule and sterile lemma development. In *Osmads34/pap2* mutants, sterile lemmas are elongated to form leaf-like or lemma-like organs [17-19]. The results from evolution and sequence analyses of *OsMADS34/PAP2* support the hypothesis that the sterile lemmas of rice originate from the degenerated floret lemma, which is named the rudimentary lemma [19]. *LONG STERILE LEMMA1* (*G1*)/*ELONGATED EMPTY GLUME* (*ELE*) encodes a DUF640-containing protein that determines the identity of the sterile lemmas. The mutation of *G1/ELE* induces sterile lemmas to become lemma-like organs [20-21]. Interestingly, natural mutations in the sterile lemmas cause similar homeotic conversions in the genome of allotetraploid *Oryza grandiglumis*, which suggests that sterile lemmas might 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 affects the agronomic character of rice remain unclear. In this study, *long sterile lemma 2* (*Isl2*), a new strong mutant allele of *G1*, was identified in the ZH11 background. We mapped *Isl2*, analysed the 3-D structure of the LSL2 protein, and found that the *Isl2* protein harbours a one-amino-acid change, namely, the mutation of serine (Ser) 79 to proline (Pro), and this change is likely to alter the structure of the LSL2 protein. We also performed molecular cloning of *Isl2* and analysed the agronomic characteristics of the *Isl2* mutant. Together, the results indicate that *Isl2* has specific value in rice crossbreeding.

## Methods

### Plant materials

*Indica* rice CO39 and *japonica* ZH11 were provided by the Plant Immunity Center at Fujian Agriculture and Forestry University and were preserved at the Rice Research Institute at Fujian Academy of Agricultural Sciences (China). The long sterile lemma mutant in the ZH11 background was screened from the M<sub>2</sub> population treated with ethyl methanesulfonate (EMS) and named *long sterile lemma 2* (*Isl2*). Approximately 800 plants from the M<sub>1</sub> population and 6000 plants from the M<sub>2</sub> population were field-grown at Fuzhou Experimental Station at Fujian Academy of Agricultural Sciences in 2016 and 2017, respectively.

In the summer of 2018, the *Isl2* mutant was hybridized with the rice cultivars CO39 and ZH11 as the pollen donors. The F<sub>1</sub> seeds were sown at Sanya (18.14 northern latitude, 109.31 east longitude) Experimental Station in Hainan Province in the spring, and F<sub>2</sub> seeds were harvested. The F<sub>2</sub> seeds *Isl2* and ZH11 were planted at Fuzhou (26.08 northern latitude, 119.28 east longitude) Experimental Station in Fujian Province in the summer of 2019. The 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 the mutant versus the wild-type plants were examined after maturity.

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

### **Construction of the mapping population**

The *Is12* mutant (*japonica*) was hybridized with CO39 (*indica*) to produce a mapping population. The F<sub>2</sub> population was constructed through self-crossing of the F<sub>1</sub> population, and 1084 mutant-phenotype plants in the F<sub>2</sub> 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. Nipponbare) [22] and *indica* (cv. 93-11) [23]. First, the bacterial artificial chromosome (BAC) clone sequences of *japonica* and *indica* were compared, and Primer premier 5.0 was then 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**

Plant DNA was extracted from frozen leaves of rice plants using the CTAB method [24], with minor modifications. For PCR amplification, every 20- $\mu$ L reaction mixture contained 30 ng of DNA, 0.4  $\mu$ M of each primer, and 2 $\times$  Es Tag MasterMix (Dye). The amplification procedure was performed using the following program: 2 min at 94°C, 33 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and 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**

Markers associated with target genes were identified by bulk segregant analysis (BSA). DNA from the leaves of 15 randomly selected mutant plants of the F<sub>2</sub> population was used to construct a mutant DNA library. Linkage was detected based on the distribution of SSR markers in the rice genome through an analysis of DNA extracted from the *Is12* mutant and CO39 (used as a control). The bands of markers linked to the mutant genes were the same as those found with the *Is12* mutant.

### **Molecular mapping of the *Is12* gene**

The band types of the mutants (*Is12 Is12*) and (*LSL2LSL2*) were denoted 1 and 3, respectively, and 2 was used to represent a heterozygote (*Is12 LSL2*). Linkage analysis between the *Is12* locus and the SSR

markers was performed using MAPMAKER version 3.0 software [26]. The map distances were estimated using MapDraw V2.1 [27]. The linkage map obtained in this study was almost equal to that reported previously [28].

First, 326 SSR markers were selected from the rice molecular map for analysis of the polymorphism between *Is/2* and CO39 [29]. Among these markers, 205 pairs showed polymorphism, and based on these 205 markers, 15 mutant strains and 15 normal strains were selected from the F<sub>2</sub> population for linkage analysis of the *Is/2* locus. Second, to delineate the gene to a smaller region, we identified 1084 mutants from the F<sub>2</sub> population of *Is/2* × CO39, and indel markers from the open rice genome sequences were designed to predict the likelihood of polymorphisms between *Is/2* 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 by 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 *Is/2* and *LSL2* were used for complete alignment with Clustal X version 1.81. The 3-D structures of the *Is/2* and *LSL2* proteins were predicted and analysed (<https://swissmodel.expasy.org/>). A haplotype analysis of *Is/2* 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 the genome-editing mutations of the target gene in the regenerated plants were evaluated. The chromosomal deletions and insertions were detected by PCR using primers located in gene target sites. The PCR products were selected from the transgenic CRISPR-edited lines for sequencing to identify specific mutations. Double peaks were resolved using the degenerate sequence decoding method [31]. The primers used in the CRISPR/Cas9 study are listed in Supplementary Table 1.

### RNA extraction and expression status of *LSL2* by RT-qPCR

RNA extraction was performed using kits (Magen, IF210200) according to the manufacturer's protocols. Total RNA quality detection was performed with a Nanodrop for determining the RNA concentration;

nondenaturing agarose electrophoresis for determining RNA integrity.

To verify the expression status of *LSL2*, empty glumes and lemma/palea were selected for qPCR cDNA was synthesized by reverse transcription. QPCR primers were designed using Primer5 software, and primer specificity was evaluated by blasting primer sequences against the NCBI database (qPCR-F -5'CCGGGACTGGCAGACCTTCAC-3', qPCR-R-5'GTCGCATCGCCGTCGTTCA3'). The constitutively expressed rice gene ubiquitin conjugating enzyme E2 was used as a reference gene for normalization [32]. Three technical replicates for each of three biological replicates were performed.

### **Measurement of the germination rates**

Each rice material was incubated in a plant-light incubator for 24 hours; 100 seeds of each material were germinated, and this process was repeated four 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 performed at 25°C. The number of germinated seeds was recorded after 2 days, and the germination rate was continuously recorded until the 7th day. The moisture and temperature conditions were maintained.

## **Results**

### **Main agronomic characteristics of *ls/2***

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 (ls/2)* mutant in the ZH11 background. Phenotypic comparisons between the *ls/2* mutant and wild-type ZH11 plants are presented in Table 1. The results showed no significant differences in the major agronomic traits, including the plant height, panicle length, number of effective panicles, spikelets per panicle, seed-setting rate, 1,000-grain weight, grain length and grain width.

### **Phenotypic observations and analysis of the *ls/2* mutant**

At the vegetative stage, the phenotypes of the ZH11 and *ls/2* plants were indistinguishable, but their spikelets displayed different phenotypes from the boot stage to the mature stage (Table 1 and Fig. 1a, b). The sterile lemma of the *ls/2* mutants was markedly longer than that of ZH11, although other components of the spikelet were the same (Fig. 1a, b). Interestingly, no significant difference in the grain size or brown rice size was found between *ls/2* and ZH11 after maturation (Table 1 and Fig. 1c, d).

We compared the germination rates of the *ls/2* and ZH11 seeds. On the second day, the wild-type ZH11 plants started sprouting (69.3%), but the *ls/2* mutant had barely begun to germinate (2.3%) (Fig. 2 and Table 2). Compared with the wild-type plants, the *ls/2* mutants showed clearly reduced germination rates from the second day to the fourth day (Table 2).

### **Genetic analysis of the *ls/2* mutant**

To determine whether the *ls/2* mutant is caused by a single gene, we then crossed the *ls/2* mutant with ZH11. The F<sub>1</sub> generation showed normal phenotypes, and the F<sub>2</sub> population exhibited Mendelian segregation (Table 3). Indeed, the segregation between the wild-type and mutant plants corresponded to a 3:1 segregation ratio in the two F<sub>2</sub> populations ( $\chi^2=0.124 \ll 0.462$ ,  $P>0.5$ ), which indicated that the *ls/2* mutant phenotype is controlled by a single recessive gene.

### Initial localization of the *ls/2* gene

To determine which gene mutation causes the *ls/2* phenotype, we then mapped the *ls/2* gene. Two SSR markers located on rice chromosome 7, RM4584 and RM2006, were found to be associated with mutant traits in 193 F<sub>2</sub> individuals. Based on the recombination frequency, the genetic distance between RM4584 and RM2006 was calculated to equal 28.8 cM. Therefore, *ls/2* is located in a 28.8-cM region on chromosome 7 flanked by the SSR markers RM4584 and RM2006 (Fig. 3a).

### Fine mapping of the *ls/2* gene

To delineate the gene to a smaller region, an accurate map between RM4584 and RM2006 was constructed using published markers (Table 4). Through genetic linkage analysis, the *ls/2* gene was mapped between the molecular markers RM8059 and RM427, with a distance of 7.6 cM (Fig. 3b). For further mapping, all recombinant genes were genotyped using nine polymorphic markers (Table 4). The results showed that the *ls/2* gene is located between the molecular markers Indel7-13 and Indel7-15, with a physical distance of 205 kb (Fig. 3c and Table 4). For the fine mapping of the *ls/2* gene, seven polymorphic indel markers for recombinant screening (Table 4) detected one, one, three, three, six, seven and 11 recombinant plants (Fig. 3d). Thus, we precisely localized the *ls/2* 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 ([LOC\\_Os07g04660](#), [LOC\\_Os07g04670](#), [LOC\\_Os07g04690](#), and [LOC\\_Os07g04700](#)) were annotated 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, and [LOC\\_Os07g04670](#), [LOC\\_Os07g04690](#) and [LOC\\_Os07g04700](#) encode a DUF640 domain-containing protein, UDP-arabinose 4-epimerase 1, and an MYB family transcription factor, respectively.

### Sequence analyses of the *ls/2* gene

To analyse which gene causes the mutant phenotype, we sequenced the above-mentioned four genes in ZH11 and *ls/2* and found only a single 1-bp change (T to C) in [LOC\\_Os07g04670](#) between the wild-type ZH11 and *ls/2* mutant plants. No other differences in the sequences of the three other genes were observed. Thus, we speculated that the [LOC\\_Os07g04670](#) locus corresponds to *ls/2*. Interestingly, the *G1/ELE* gene, which encodes a DUF640 domain-containing protein, is present in this locus [20]. Based on

the results from phenotypic similarity and localization analyses, we hypothesized that the long sterile lemma phenotype of *ls2* might be caused by functional changes in the product of the LOC\_Os07g04670 locus. These results suggest that the *ls2* gene might be allelic with *G1/ELE*.

The analysis of the open reading fragment (ORF) of the *LSL2* gene (LOC\_Os07g04670) showed one exon and no intron. *ls2* is a 1-bp mutant that resulted in the exchange of a serine (Ser) for a proline (Pro) (Fig. 4). Ser is a polar amino acid, whereas Pro is nonpolar. Thus, this mutation might alter the function of a protein.

### **The *ls2* gene is responsible for the long sterile lemma phenotype**

To confirm that the mutation phenotype can be attributed to *ls2*, we examined whether the 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 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 exhibited a long sterile lemma phenotype (Fig. 5), which indicated that the knockout of *LSL2* in ZH11 leads to the long sterile lemma mutation phenotype.

### **qPCR confirms expression status of *LSL2***

To verify the expression status of *LSL2*, empty glumes and lemma/palea were selected for qPCR. The results showed that the *LSL2* was expressed significantly differently in empty glumes and lemma/palea, and the expression level in empty glumes was significantly higher than that in lemma/palea (Fig. 6).

### **Analyses of 3-D structures between the *LSL2* protein and the *Isl2* protein**

Further simulations of the 3-D structures of the proteins revealed changes between the *Isl2* and *LSL2* proteins (Fig. 5). Moreover, the change in residue 79 of *LSL2* from Ser to Pro induced a significant change in the protein structure (Fig. 7).

### **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 [33] and found 492 haplotypes for the *LSL2* gene, including 49 haplotypes among more than 15 rice resource materials (Supplementary Table 2). However, no haplotype or SNP was found for the *ls2* mutant in the 3,000 sequenced rice genomes.

## **Discussion**

### **Mechanism controlling the development of empty glumes and lemmas**

The molecular mechanism that determines the development of the lemma differs from that involved in the development of the empty glume [13]. Two genes reportedly determine the identities of empty glumes: *G1/ELE* and *OsMADS34/PAP2*. The *G1/ELE* gene is key for maintaining the identities of empty glumes, and Yoshida et al. identified glumes as remnants of two lower reduced florets and named them the sterile lemma [20]. Similarly, *OsMADS34/PAP2* is also a key regulator of empty glume development [19]. Through an 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]. *OsMADS1* specifies the identities of the lemma and palea and distinguished the empty glume from the lemma/palea [34,35].

However, there are several controversial interpretations regarding the identities of empty glumes, including true glumes and lemmas. Yoshida et al. regarded empty glumes as remnants of two lower reduced florets [20], and similarly, Lin et al. hypothesized that empty glumes originate from lemmas and named these rudimentary lemmas [19]. Most likely, the research progress in the development of lemmas and further molecular evidence might provide clues for determining the identities of empty glumes. The key genes controlling the lemma identity need to be identified and cloned, and their expression in empty glumes should be analysed.

In this study, we knocked out the *LSL2* gene using the CRISPR/Cas9 gene editing system, and three independent lines were obtained for the target sites: Line 1 carries a 1-bp insertion, Line 2 harbours a 2-bp deletion, and Line 3 carries a 1-bp deletion. Further analysis of the panicle characteristics showed that all three lines exhibited a long sterile lemma phenotype (Fig. 5 and Table 5), and this phenotype was the same as that of the *ls/2* mutant. Therefore, the *ls/2* gene was found to be responsible for the long sterile lemma phenotype.

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-based identification of glumes.

### **Genetic and evolutionary analyses of the *LSL2* gene**

A 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 *ls/2* mutant, which contains a T-to-C mutation, was found in the 3,000 sequenced rice genomes. We speculate that a mutation at this site would be strongly selected against in natural selection and would only be the result of manual selection. For example, the phenotypes of *ls/2* might be inconsistent with the expectations of breeders; therefore, this mutation was gradually eliminated by manual selection.

Because simulations of the 3-D structures of *LSL2* and *ls/2* showed that the T-to-C amino acid change alters the protein structure (Fig. 7), we speculate that this change might affect the specific function of *LSL2*, such as its 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, whether it affects the internal characteristics of rice remains unclear. The comparison of the germination rates of *lsl2* and ZH11 seeds revealed that the *lsl2* mutant exhibited obviously reduced germination rates from the second day to the fourth day (Table 2). We propose that the most likely reason for this difference is that the longer sterile lemma of *lsl2* might 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, and these effects cause economic losses to different degrees [36]. In this study, we found that the *lsl2* mutation reduced the damage induced 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-assisted selection.

## Conclusions

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

## Declarations

### Acknowledgements

Not applicable.

### Authors' contributions

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

### Funding

The work was supported by the Special Fund for Agro-scientific Research in the Public Interest of Fujian Province (No. 2020R11010016-3), the Youth Technology Innovation Team of Fujian Academy of Agricultural Sciences (No. STIT2017-3-3) and the Fujian Provincial Natural Science Foundation of China (No. 2019J01011040). These three funders provided financial support to our study.

## 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.

## Author details

Rice Research Institute, Fujian Academy of Agricultural Sciences, Fujian High Quality Rice Research & Development Center, Fuzhou 350019, China

## Abbreviations

## References

1. Ronse De Craene L. Understanding the role of floral development in the evolution of angiosperm flowers: clarifications from a historical and physico-dynamic perspective. *J Plant Res.* 2018;131:367-393.
2. Coen ES, Meyerowitz EM. The war of the whorls: genetic interactions controlling flower development. *Nature.* 1991;353:31-37
3. Theissen G, Saedler H. Plant biology: Floral quartets. *Nature.* 2001;409:469-471.
4. Irish V. The ABC model of floral development *Curr Biol.* 2017;27:R887-R890.
5. Ali Z, Raza Q, Atif RM, Aslam U, Ajmal M, Chung G. Genetic and molecular control of floral organ identity in cereals. *Int J Mol Sci.* 2019; 20(11):2743
6. Wang HM, Tong CG, Jang S. Current progress in orchid flowering/flower development research. *Plant Signal Behav.* 2017; 12(5):e1322245.
7. Thomson B, Wellmer F. Molecular Regulation of Flower Development. *Curr Top Dev Biol.* 2019;131:185-210.
8. Nagasawa N, Miyoshi M, Sano Y, Satoh H, Hirano H, Sakai H, Nagato Y. *SUPERWOMAN 1* and *DROOPING LEAF* genes control floral organ identity in rice. *Development.* 2003;130:705-718.

9. Yamaguchi T, Lee DY, Miyao A, Hirochika H, An G, Hirano HY. Functional diversification of the two C-class *MADS* box genes *OSMADS3* and *OSMADS58* in *Oryza sativa*. *Plant Cell*. 2006;18:15-28.
10. Dreni L, Jacchia S, Fornara F, Fornari M, Ouwerkerk PB, An G, Colombo L, Kater MM. The D-lineage *MADS*-box gene *OsMADS13* controls ovule identity in rice. *Plant J*. 2007;2:690-699.
11. Xu W, Tao JH, Chen MJ, Dreni L, Luo ZJ, Hu Y, Liang WQ, Zhang DB. Interactions between *FLORAL ORGAN NUMBER4* and floral homeotic genes in regulating rice flower development. *J Exp Bot*. 2017;68:483-498.
12. HuY, Liang WQ, Yin CS, Yang XL, Ping BZ, Li AX, Jia Ru, Chen MJ, Luo ZJ, Cai Q, Zhao XX, Zhang DB, Yuan Z. Interactions of *OsMADS1* with floral homeotic genes in rice flower development. *Mol Plant*. 2015;8(9):1366-1384.
13. Liu MJ, Li HF, Su YL, Li WQ, Shi CH. *G1/ELE* functions in the development of rice lemmas in addition to determining identities of empty glumes. *Front Plant Sci*. 2016;7:1006.
14. Yoshida H, Nagato Y. Flower development in rice. *J Exp Bot*. 2011;62:4719-4730.
15. Lombardo F, Yoshida H. Interpreting lemma and palea homologies: a point of view from rice floral mutants. *Front Plant Sci*. 2015;6:61.
16. Cui RF, Han JK, Zhao SZ, Su KM, Wu F, Du XQ, Xu QJ, Chong K, Theissen G, Meng Z. Functional conservation and diversification of class E floral homeotic genes in rice (*Oryza sativa*). *Plant J*. 2010;61:767-781.
17. Kobayashi K, Maekawa M, Miyao A, Hirochika H, Kyoizuka J. *PANICLE PHYTOMER2 (PAP2)*, encoding a *SEPALLATA* subfamily *MADS*-box protein, positively controls spikelet meristem identity in rice. *Plant Cell Physiol*. 2010;51:47-57.
18. Gao XC, Liang WQ, Yin CS, Ji SM, Wang HM, Su X, Guo C, Kong HZ, Xue HW, Zhang DB. The *SEPALLATA*-like gene *OsMADS34* is required for rice inflorescence and spikelet development. *Plant Physiol*. 2010;53:728-740.
19. Lin XL, Wu F, Du XQ, Shi XW, Liu Y, Liu SJ, Hu YX, Theissen G, Meng Z. The pleiotropic *SEPALLATA*-like gene *OsMADS34* reveals that the 'empty glumes' of rice (*Oryza sativa*) spikelets are in fact rudimentary lemmas. *New Phytol*. 2014;202:689-702.
20. Yoshida A, Suzuki T, Tanaka W, Hirano HY. The homeotic gene long sterile lemma (*G1*) specifies sterile lemma identity in the rice spikelet. *Proc Natl Acad Sci*. 2009;106:20103-20108.
21. Hong LL, Qian Q, Zhu KM, Tang DX, Huang ZJ, Gao L, Li M, Gu MH, Cheng ZK. *EL* restrains empty glumes from developing into lemmas. *J Genet Genomics*. 2010;37:101-115.
22. Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D, Hutchison D, Martin C, Katagiri F, Lange BM, Moughamer T, Xia Y, Budworth P, Zhong J, Miguel T, Paszkowski U, Zhang S, Colbert M, Sun WL, Chen L, Cooper B, Park S, Wood TC, Mao L, Quail P, Wing R, Dean R, Yu Y, Zharkikh A, Shen R, Sahasrabudhe S, Thomas A, Cannings R, Gutin A, Pruss D, Reid J, Tavtigian S, Mitchell J, Eldredge G, Scholl T, Miller RM, Bhatnagar S, Adey N, Rubano T, Tusneem N, Robinson R, Feldhaus J, Macalima T, Oliphant A, Briggs S. A draft sequence of the rice genome (*Oryza sativa L ssp Japonica*). *Science*. 2002;96:920-100.

23. Yu J, Hu SN, Wang J, Wong GK S, Li SG, Liu B, Deng YJ, Dai L, Zhou Y, Zhang XQ, Cao M L, Liu J, Sun JD, Tang JB, Chen YJ, Huang XB, Lin W, Ye C, Tong W, Cong LJ, Geng JN, Han YJ, Li L, Li W, Hu GQ, Huang XG, Li WJ, Li J, Liu ZW, Li L, Liu JP, Q, QH, Liu JS, Li L, Li T, Wang XG, Lu H, Wu TT, Zhu M, Ni PX, Han H, Dong W, Ren XY, Feng XL, Cui P, Li XR, Wang H, Xu X, Zhai WX, Xu Z, Zhang J S, He SJ, Zhang JG, Xu JC, Zhang KL, Zheng XW, Dong JH, Zeng WY, Tao L, Ye J, Tan J, Ren XD, Chen XW, He J, Liu DF, Tian W, Tian CG, Xia HG, Bao QY, Li G, Gao H, Cao T, Wang J, Zhao WM, Li P, Chen W, Wang XD, Zhang Y, Hu J F, Wang J, Liu S, Yang J, Zhang G , Xiong YQ, Li ZJ, Mao L, Zhou CS, Zhu Z, Chen RS, Hao BL, Zheng WM, Chen SY, Guo W, Li GJ, Liu SQ, Tao M, Wang J, Zhu LH, Yuan LP Yang, HM. A draft sequence of the rice genome (*Oryza sativa L ssp Indica*). Science. 2002;296:79-92.
24. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA Nucleic Acids Res. 1980;8:4321-4325.
25. Panaud O, Chen X, Mccouch SR. Development of microsatellite and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa L*). Mol Gen Genet. 1996;252: 597-607.
26. Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newberg LA. Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics. 1987;1:174-181.
27. Liu HR, Meng JL. MapDraw: amicrosoft excelmacrofor drawing genetic linkage maps based on given genetic linkage data. Hereditas (Beijing). 2003;25:317-321.
28. Rahman ML, Chu SH, Choi MS, Qiao YL, Jiang WZ, Piao RH, Khanam S, Cho YI, Jeung JU, Jena KK, Koh HJ. Identification of QTLs for some agronomic traits in rice using an introgression line from *Oryza minuta*. Mol Cell. 2007;24:16-26.
29. Mccouch SR, Teytelma, L, Xu YB, Lobos KB, Clare K, Walton M, Fu BY, Maghirang R, Li ZK, Xing YZ, Zhang QF, Kono I, Yano M, Fjellstrom R, Declerck G, Schneider D, Cartinhour S, Ware D, Stein L. Development and mapping of 2240 new SSR markers for rice (*Oryza setiva L*). DNA R 2002;9:257-279.
30. Xie KB, Zhang JW, Yang YN. Genome-wide prediction of highly specific guide RNA spacers for CRISPR-Cas9-mediated genome editing in model plants and major crops. Mol Plant. 2014;7:923-926.
31. Ma XL, Chen LT, Zhu QL, Chen YL, Liu YG. Rapid decoding of sequence-specific nuclease-induced heterozygous and biallelic mutations by direct sequencing of PCR. products. Mol Plant. 2015;8:1285-1287.
32. Jain M, Nijhawan A, Tyagi AK, Khurana JP. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Bioph Res Co. 2006; 345:646-651.
33. Li ZK, Fu BY, Gao YM, Wang WS, Xu JL, Zhang F, Zhao XQ, Zheng TQ, Zhou YL, Zhang G, Tai SS, Xu JB, Hu Ws, Yang M, Niu YC, Wang M, Li YH, Bian LL, Han XL, Li J, Liu X, Wang B. The 3,000 rice genomes project. Gigascience. 2014;3:7.
34. Prasad K, Parameswaran S, Vijayraghavan Usha. *OsMADS1*, a rice MADS-box factor, controls differentiation of specific cell types in the lemma and palea and is an early-acting regulator of inner

floral organs. The Plant Journal. 2005;43(6):915-928.

35. Wang L, Zeng XQ, Zhuang H, Shen YL, Chen H, Wang ZW, Long JC, Ling YH, He GH, Li YF. Ectopic expression of *OsMADS1* caused dwarfism and spikelet alteration in rice. Plant Growth Regulation. 2017;81(3):433-442.
36. Wang Z, Tang H. Effects of exogenous ABA on panicle sprouting of F<sub>1</sub> in hybrid rice seed p Acta Agronomica Sinica. 2000;26(1):59-64.

## Tables

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

Traits	ZH11	<i>lsl2</i>
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 panicles	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

\* P<0.05 and \*\* P<0.01 for the difference between ZH11 and *lsl2*. The data were derived from the trial performed at Fuzhou Experimental Station in October 2019.

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

Number of days	1d	2d**	3d**	4d *	5d	6d	7d
Name of the material							
Germination rate of ZH11 (%)	0	69.3	94.8	95.3	95.8	96.8	96.8
Germination rate of <i>lsl2</i> (%)	0	2.3	63.5	82.5	94.6	95.6	95.6

\* P<0.05 and \*\* P<0.01 for the difference between ZH11 and *lsl2*.

**Table 3.** Segregations of the F<sub>2</sub> population produced by crossing the *lsl2* mutant

Crosses	F <sub>1</sub> phenotype	F <sub>2</sub> population			$\chi^2$ (3:1)	P
		Wild-type plants	Mutant plants	Total plants		
<i>lsl2</i> /ZH11	Normal type	180	57	237	0.462*	0.5-0.75
ZH11/ <i>lsl2</i>	Normal type	198	64	262	0.124*	>0.9

\* Denotes the segregation ratio of normal 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 the forward primer	Sequence of the reverse primer
RM8059	GGAAAGACCAGTTTAGAGCAATGG	AGCTAGATCCCTTGTTTCACACG
RM427	TACTAGCTCTGCCCTGACC	TGATGAGAGTTGGTTGCGAG
RM4098	CGTTTGGATGAAGAAGAAGA	AGTGTTTCGTTTCGGATTAGA
Indel7-2	CAGATATGATGTTCTTGCCCTTGC	GCTTGCCAGATCACCTACCTACC
Indel7-3	CGGAGCTGTTGCCGTTCTGC	CGATGTGCCATGTCAGGATGACC
Indel7-5	CCTACCGCGTCATTACATGC	GACAAGATCGACAGCCGCTACG
Indel7-6	TACTCACACACTGACTGACACG	TCTCGTCGGAGAAGAAGATGAGC
Indel7-9	CACTATGGATCTTGGTGGTCAAGG	TGCTATCTGCTACCGTCAACACG
Indel7-13	GTAGGACATGAAGGCGGCTAGG	ATTCCTGCCACTGCACACC
Indel7-15	CGTCCATATCAAACCTCTTCTTCC	GTAACATTCCCTCCCGAACTCC
Indel7-16	GGTGCAGACTACCTAAATATGACG	GTAACCGATGGCTTAGAGTCC
Indel7-19	AACGGGAGATCACAGGAATTTGC	GTGTTTCGACTCGTCTCCATTTTCG
Indel7-22	ACAGTGAAAGCCACTACCAT	CTGACTGGGTGTCCATATT
Indel7-27	TAGGTGCAACTTCTTGAAGTG	GATCCCCTGTTTCATTTGTAATT
Indel7-30	AGGGGCGCACAGCGGGGAGGGTC	TCAATCCACGGAATCCACGAC
Indel7-35	GATTTCAGAAGATGTTTGGG	GGTTTCCAGTTTCTGTCTT
Indel7-38	TGATTTTATCCTCGTCTTCC	AACATGCGCATATGTAAGT
Indel7-40	TCTCTTCTCTCTTGCTTCTC	ATGTCAATTTGATGGGATGT
Indel7-42	TGAAAAGAAGTCAATGCT	TTGAATCACCACAATTTAGC

**Table 5.** Mutation site of three targeted mutant lines

Line	Target type	Mutation site
Line 1	gRNAs	ACTGGCAGACCTTCACGCAGTTACCTCGCCGCGCACCCGCCGC (1-bp insertion)
Line 2	gRNAs	ACTGGCAGACCTTCACGCAGT--CTCGCCGCGCACCCGCCGC (2-bp deletion)
Line 3	gRNAs	ACTGGCAGACCTTCACGCAGT-CCTCGCCGCGCACCCGCCGC (1-bp deletion)

## Figures



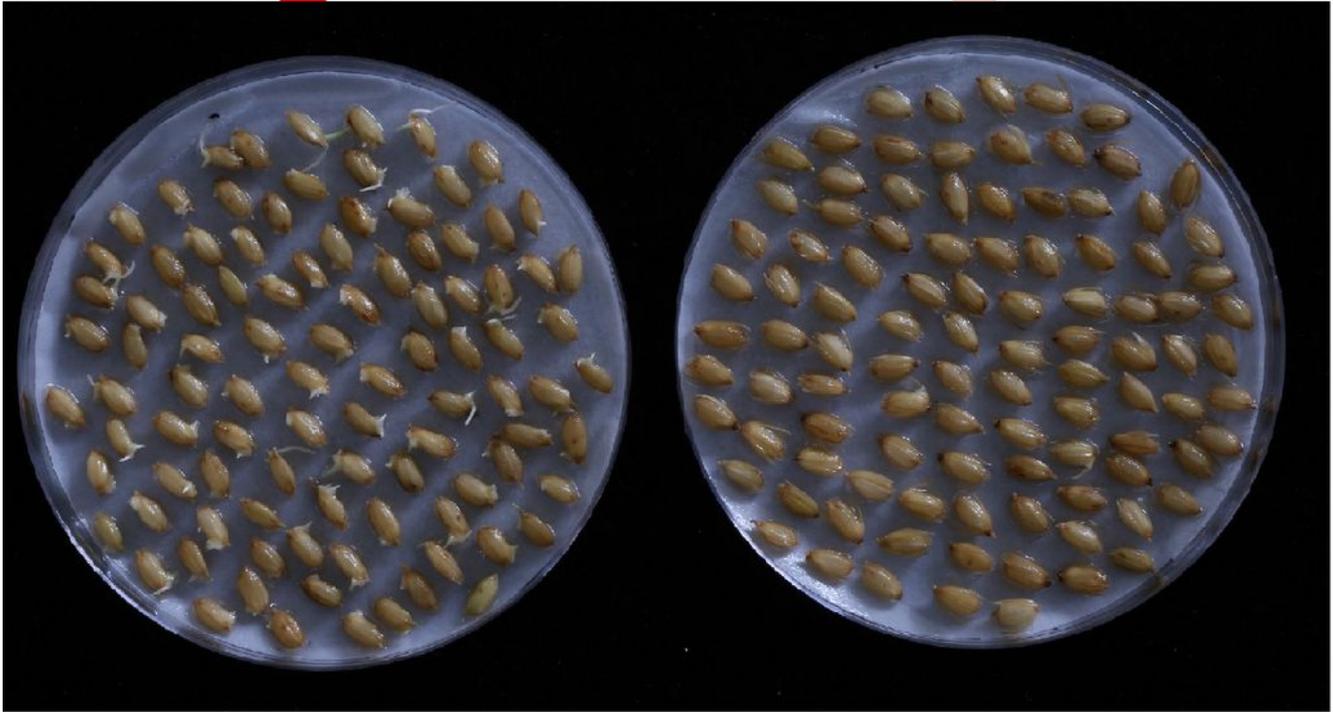
**Figure 1**

Phenotypes of ZH11 and the *lsl2* mutant. a: Grain phenotypes of ZH11; b: grain phenotypes of the *lsl2* mutant; c: brown rice phenotypes of ZH11; d: brown rice phenotypes of the *lsl2* mutant. The *lsl2* mutant has a markedly longer sterile lemma compared with ZH11, and no significant difference in the grain size or shape were found between the *lsl2* mutant and ZH11.



**ZH11**

*lsl2*

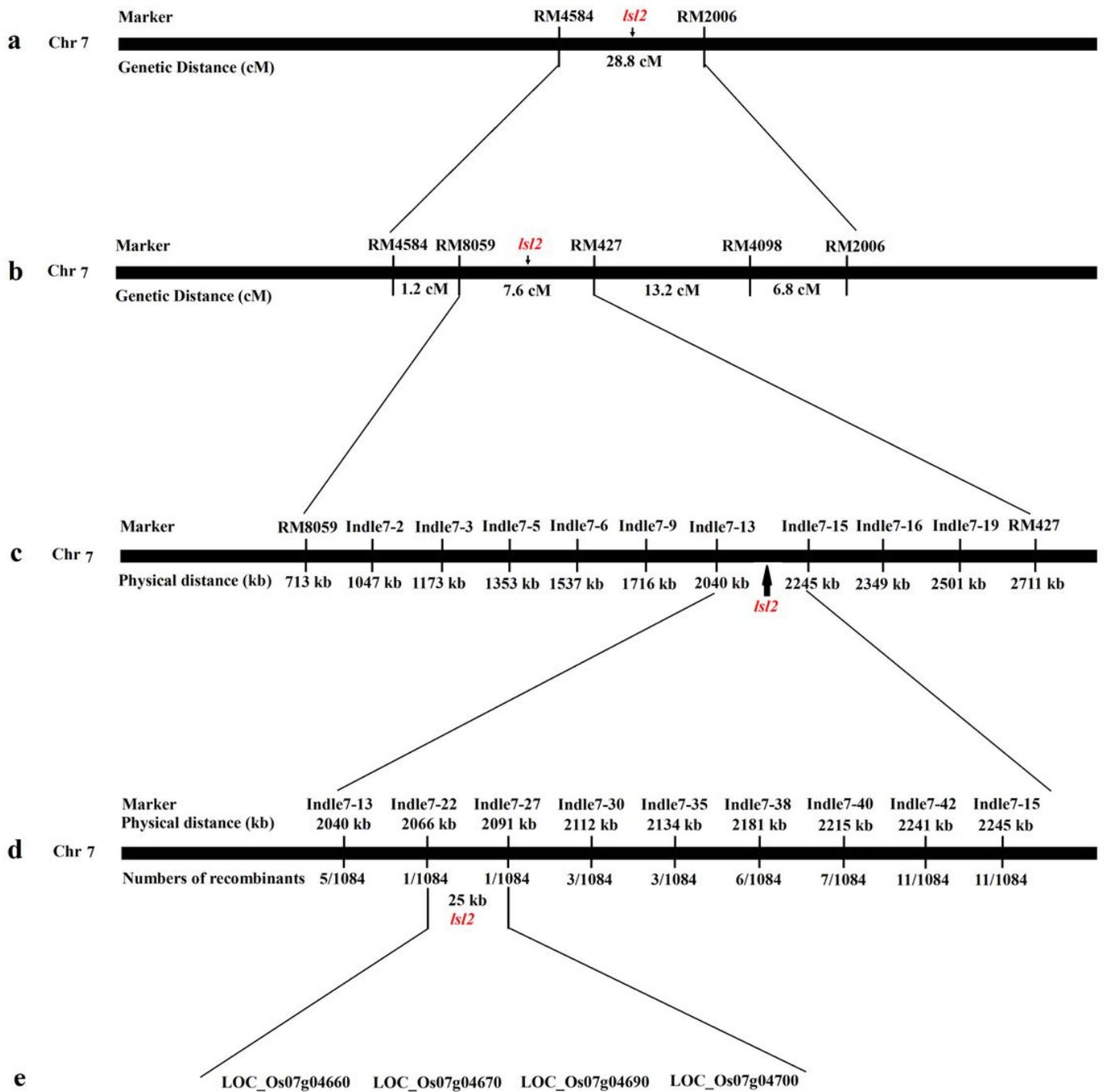


**ZH11**

*lsl2*

**Figure 2**

Comparison of the germination rate between *lsl2* mutant and ZH11 seeds. ZH11 showed a higher germination rate than the *lsl2* mutant starting on the second day.



**Figure 3**

Genetic and physical maps of the *Isl2* gene. a: Primary mapping of the *Isl2* gene. The gene was mapped to the region between the markers RM4584 and RM2006. b: Further mapping of the *Isl2* gene. The gene was mapped to the region between the markers RM8059 and RM427. c: Fine mapping of the *Isl2* gene. The gene was mapped to the region between the markers Indel7-13 and Indel7-15. d: High-resolution mapping of *Isl2*. The *Isl2* gene was localized to a 25.0-kb region between the markers Indel7-22 and

Indel7-27, and the recombinant number between the markers and the target gene is indicated under the linkage map. e: Candidate genes in the 25.0-kb target region.

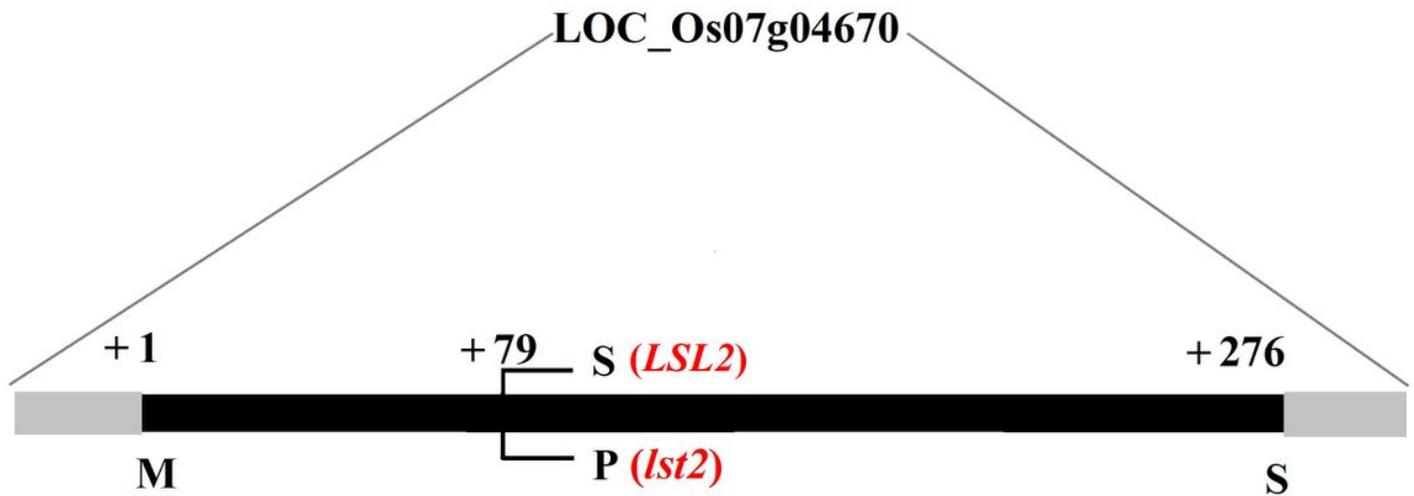


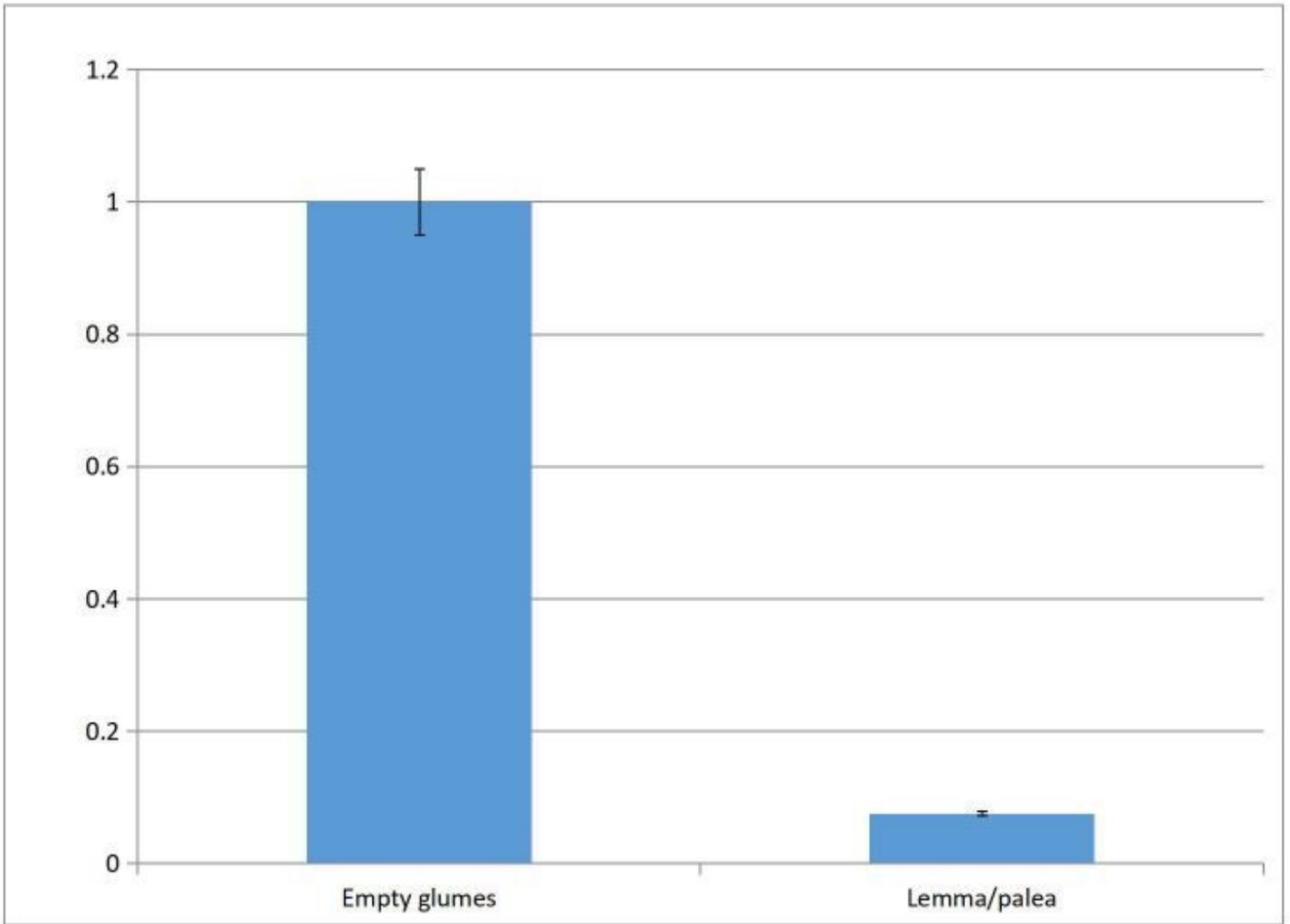
Figure 4

Structural comparison between the LSL2 and Isl2 proteins. Only one amino acid substitution (S79P) was found between LSL2 and Isl2.



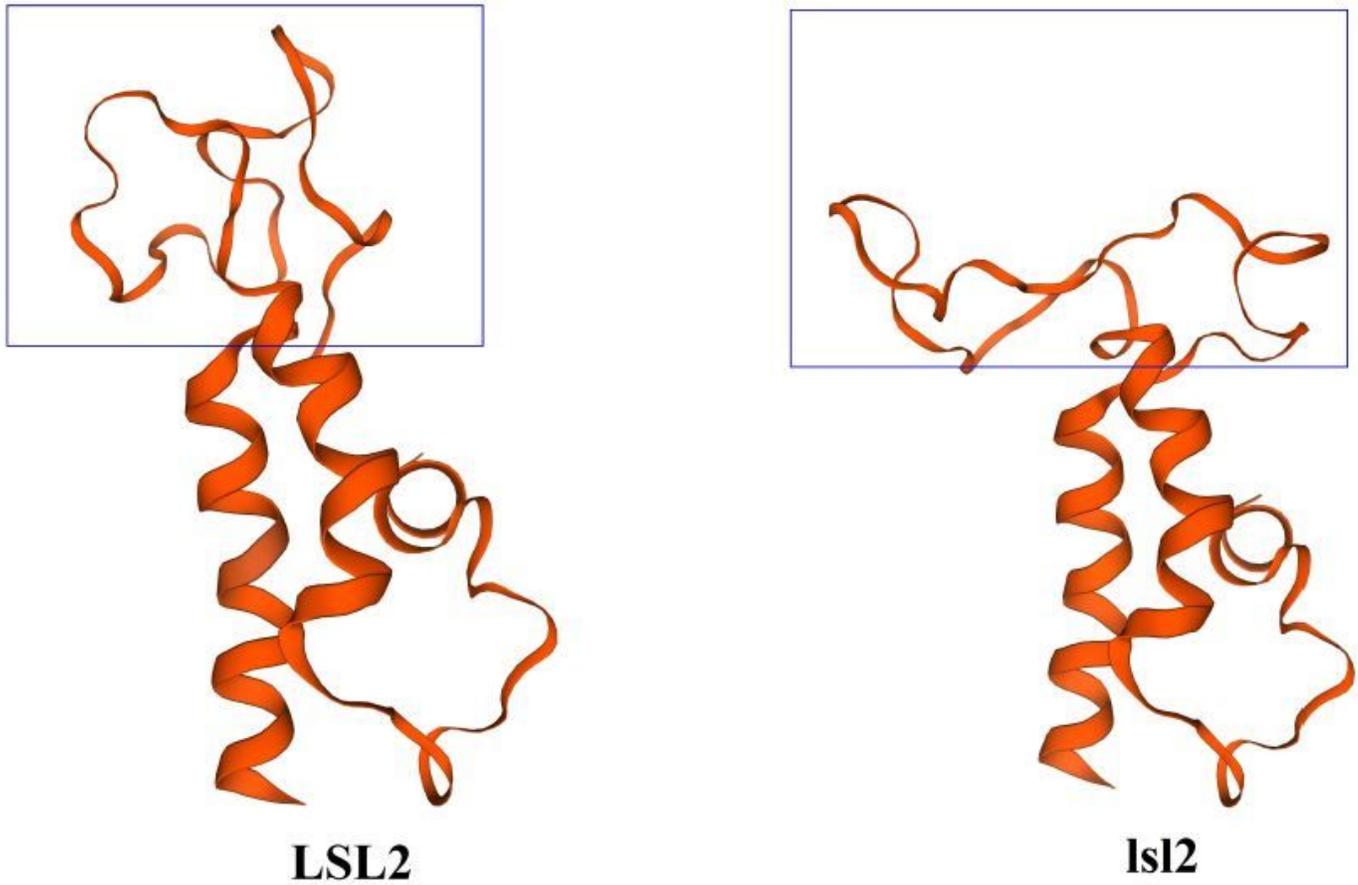
Figure 5

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



**Figure 6**

The expression status of LSL2 between empty glumes and lemma/palea. The expression level of LSL2 in empty glumes was significantly higher than that in lemma/palea.



**Figure 7**

3-D structures of the LSL2 and Isl2 proteins. Significant structural changes were detected based on the Swiss-Model. In Isl2, the 79th residue of the LSL2 protein is changed from a serine (S) to a proline (P), and this change significantly alters the structure. The blue square indicates the site of the change in the 79th amino acid.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTables.docx](#)