

Research Paper

Identification and fine mapping of lesion mimic mutant *spl36* in rice (*Oryza sativa* L.)

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In the absence of pathogen attack, lesion mimic mutants (LMMs) in plants undergo spontaneous cell death and develop necrosis or apoptosis-like lesions on the leaves or sheath, resembling symptoms of hypersensitive response. In-depth research has been conducted on LMMs, especially regarding the molecular mechanisms underlying programmed cell death and disease resistance. In this study, the *spotted leaf 36* (*spl36*) mutant was identified as a typical LMM, showing lesions on both the leaf blade and leaf sheath. The formation of lesions was found to be caused by cell death accompanied by accumulation of hydrogen peroxide and degradation of chloroplasts. Compared with wild-type, the main agronomic traits such as plant height, effective panicle number, panicle length, grain per panicle, seed setting rate, and 1000-grain weight of *spl36* were significantly reduced. The defence and pathogenesis-related genes *PR1a*, *PR1b*, *PR10*, and *NPRI*, were transcriptionally activated in mutant *spl36* without pathogen attack. Genetic analysis showed that the mutant phenotype was controlled by the gene *SPL36*, which was mapped to an interval of 260 kb at the end of the long arm on chromosome 11. Pathogen inoculation analysis showed that *spl36* has enhanced resistance to sheath blight, rice blast, and bacterial blight.

Key Words: lesion mimic mutant, *spl36*, disease resistance, gene mapping, rice (*Oryza sativa*).

Introduction

In the absence of pathogen attack, lesion mimic mutants (LMMs) in plants undergo spontaneous cell death and develop necrosis or apoptosis-like lesions on the leaves or sheath, resembling symptoms of hypersensitive response (HR) (Xu *et al.* 2018). HR is a typical disease-resistance response characterized by the rapid cell death at the infected site due to an incompatible interaction between plants and pathogens, and is one of the important manifestations of programmed cell death (PCD) (Xiao *et al.* 2018).

LMMs have been identified in a variety of plants including *Arabidopsis thaliana* (Igari *et al.* 2008), rice (Hurni *et al.* 2015), barley (Persson *et al.* 2009), and wheat (Sugie *et al.* 2007). A large number of LMMs have been reported since the first identification of spotted leaf (*spl*) in rice by Japanese scholars (Kiyosawa 1970). To date, more than 90 mutants have been identified in rice (<http://www.gramene.org/>). The mechanisms underlying the formation of LMMs

are very complicated and the corresponding genes are related to different biological mechanisms in plants. Mutations of disease resistance genes can result in the production of lesion mimics on leaves such as *NLS1* (Tang *et al.* 2011), *OsATI* (Mori *et al.* 2007), *OsNHI* (Chern *et al.* 2005), *OsABA2* (Liao *et al.* 2018), and *OsPti1a* (Takahashi *et al.* 2007). Besides, variable other gene families that mediating metabolic pathways (Sun *et al.* 2011, Wang *et al.* 2015a), accumulation of reactive oxygen species (ROS) and hormones (Weymann *et al.* 1995), programmed cell death (Fekih *et al.* 2015, Jin *et al.* 2015, Lin *et al.* 2012) and heat shock proteins (Qiao *et al.* 2010). Among the LMMs identified in rice, most have reported to participated in defence response to fungal pathogen *Magnaporthe oryzae* (*M. oryzae*) or/and *Rhizoctonia solani* (*Rs*), or/and bacterial pathogen *Xanthomonas oryzae* *pv.* *Oryzae* (*Xoo*) in rice. The activation of pathogenesis-related (*PR*) genes characterize the activation of a successful plant defence response, meanwhile callus and phytohormones play vital roles in defence response.

The biosynthesis and decomposition of callosin help plants resist biotic and abiotic stresses, whereas salicylic acid transmits signals in the defence response of plants. Some LMMs show accumulation of callose and an increase in salicylic acid, which can promote the expression of *PR*

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genes to enhance disease resistance (Campbell and Ronald 2005). The expression of *PR1* and probenazole-inducible protein is significantly increased in the mutants *HM47* (Feng 2015), *spl11* (Zeng *et al.* 2004), and *spl18* (Mori *et al.* 2007). *HM47* and *spl11* showed enhanced resistance to *M. oryzae* 1 and *Xoo* compared with the wild type, and the resistance to *M. oryzae* is enhanced in *spl18* as well. The identification, cloning, and functional research of rice LMMs are of great theoretical significance for exploring rice disease resistance and stress defence systems, as well as improving broad-spectrum disease resistance breeding in rice.

In this study, we identified a novel LMM *spotted leaf 36* (*spl36*) in rice from the ethyl methanesulfonic (EMS)-induced mutation library of Jinhui 10 (wild-type [WT]), the indica rice restorer line. The phenotype of *spl36* was characterized in detail and showed enhanced resistance to both fungal and bacterial pathogens in rice. A genetic study showed that *spl36* was controlled by a dominant nuclear gene and was finely mapped to an interval of 260 kb on the long arm of chromosome 11. Here, we identified a novel LMM, and mutation of the target gene *SPL36* caused enhanced resistance to three different pathogens, indicating its enhanced resistance to the defence response and potential use in rice breeding.

Materials and Methods

Materials

The stably inherited mutation *spl36* was derived from Jinhui10 treated with EMS. Tetepu, Guandong 51, Lijiang Xintuan Heigu, Sifeng 43, Hejiang 18, Dongnong 363, and Zhenlong 13 were selected as the identification varieties of the physiological races of rice blast fungus. Identification of the seven physiological species of rice blast fungus was presented by C&E Senior Experimentalist Xiaoping Yuan from China National Rice Research Institute (Hangzhou, China). The rice blast standard samples were from Hechuan, Nanchuan, Qianjiang, Yongchuan in Chongqing, and Mianning County and Xuyong County in Sichuan Province. The pathogenic strain ZHE 173 of *Xanthomonas oryzae* pv. *Oryzae*, agent of bacterial blight, was presented by Associate Professor Yuxuan Hou from China National Rice Research Institute (Hangzhou, China). The pathogenic strain RH-9 of *R. solani Kühn*, agent of sheath blight, was used in this study. It was kindly gifted by Prof. Xuebiao Pan from Yangzhou University, China.

Investigation of agronomic traits

Ten well growing WT and mutant plants were randomly selected for statistical agronomic traits. Most of the agronomic traits were examined, including the effective number of panicles, primary branches, grain number per panicle, filled number per panicle, panicle length, 1000-grain weight, and seed setting rate, and the results were analyzed using the *t*-test.

Chloroplast autofluorescence observation

At the heading stage, the second leaves of WT and mutant *spl36* were selected (the leaves of mutant were selected for LMMs) and placed in a centrifuge tube containing an embedding agent (Tissue-Tek; Sakura, Sakura City, Japan). The centrifuge tube was incubated at -20°C for 30 min and taken out when the embedding agent was completely solidified. The leaves were cut into 8 mm thick sections with a cryostat, placed on a glass slide and rinsed with ddH₂O repeatedly until there was no embedding, covered with a glass slide, and observed under a fluorescence microscope.

Determination of photosynthetic pigments and photosynthetic characteristics

At the heading stage, the WT and mutant *spl36* middle leaves were selected and photosynthetic pigments were determined according to Lichtenthaler's method (Lichtenthaler 1987). On a sunny day at 9:00 AM, five plants of both Jinhui 10 and *spl36* were randomly chosen and the net photosynthetic rate, stomatal conductance, intercellular CO₂ concentration, and transpiration rate were measured using the LI-6400 photosynthetic apparatus.

Histochemistry analysis and measurement of physiological indexes

At the heading stage, 10 randomly selected WT and *spl36* leaves of obvious phenotype and similar growth were stained with trypan blue and DAB solution according to the method of Dietrich *et al.* (1994) and Thordal-Christensen *et al.* (1997). Meanwhile, the first, second, and third leaves of the WT and *spl36* mutant were collected and used to measure ROS and proline content, as well as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) activity as previously described. The Superoxide Anion Assay Kit (A052), Proline Content Assay Kit (A107), SOD Activity Assay Kit (A001-1), CAT Assay Kit (A007-1), and POD Activity Assay Kit (A084-3) were provided by the Nanjing Jiancheng Technology Company (Nanjing, China).

Genetic analysis

The *spl36* mutant was crossed with the Indica sterile line Xinong 1A to obtain F₁, after which the phenotype of the F₁ plants was observed and the F₂ seeds were collected. The F₂ population was planted and genetic analysis was performed on the F₂ population. Ten plants of normal and lesion mimic phenotype were randomly selected from the F₂ generation population, and DNA was extracted by the modified CTAB method to construct normal and mutant gene pool. The gene pool was amplified with 96 pairs of simple sequence repeat (SSR) primers (sequence design can be found http://ensembl.gramene.org/Oryza_sativa/Tools/Blast), which were evenly distributed on all 12 chromosomes of rice and had a polymorphism between Xinong 1A and *spl36*, followed by normal gene pool linkage analysis. InDel differential primers were derived from the

Table 1. Primer sequences

Marker	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
J1212-2	GAAGCAACAGCTTTGGTGGG	GAGTTGAGGCGGCACCATAC
J1216-4	CAAGCCTCACTTGGTAACTGAGG	CGCGCTCGATAGGATCTGG
J1211-1	GGCCCGTAATGTGTTTGTCCACC	GTGATACACAGTTGCAGCATCAAGC
J0602-4	GCAATGGTAGGGTGTGATGTCTGT	CATGACCAAACGGCCTAACTTG
J1206-3	TTTAAGCAAACGAGGGCATATCC	TTGTGTGGCGTTTGGCTCC
J0602-5	CATGTGAAGTGATTCTGTTTGCACCT	CAGGGTCAGGCAGTCAGGGC
J0102-3	GTGGTGGACGAGGATCAACG	GTGGTCAGTGGTCACCATGACC
J1206-4	CGACGCGCCAGGGTAATG	GTGCTGCGTGTACTTTGGTGC
J0720-1	CCGACACCGTCTCCATTATCC	GGTCCAGTGAAGTCCAAGGAGG

differential sequence between the Indica rice sterile line Xinong 1A and Indica rice restorer line Jinhui 10. The primers are listed in [Table 1](#).

Quantitative polymerase chain reaction

RNA was extracted using a kit from Promega (Madison, WI, USA), and reverse transcription was done using a reverse transcription kit (Takara Biomedical Technology, Beijing, China). The resulting cDNA was diluted 10-fold for use. The relative expression of each gene was calculated with the $2^{-\Delta\Delta CT}$ method, and actin was selected as the internal reference gene for cDNA amplification of the related genes; there were at least three replicates for each sample. Quantitative PCR (qPCR) was mainly used for the expression analysis of some genes in the localization interval and some marker genes related to the defence response.

Resistance identification

According to the method of Huang *et al.* (1998), the rice blast resistance spectrum was determined one month after sowing. The rice sheath blight was inoculated using the toothpick embedding method of Pan *et al.* (1997). After 30 days, the rice disease stabilized and a field investigation was conducted. The disease level was determined using the previous scoring system (Zuo *et al.* 2006) with slight modification. The bacterial blight suspension production method was as follows. The medium was inoculated with activated bacteria, incubated at 28°C at 120 rpm/min, and cultivated for 24–48 h using McDonald's turbidity method to prepare a 3×10^8 cfu/mL bacterial solution concentration. At 21 days after inoculation, sterilized scissors were used to dip the above-mentioned bacteria floating liquid, cut off the leaf tip 1.5 cm diagonally, and treat five leaves of each plant. Then the incidence of WT and mutant bacterial blight was investigated.

Results

Phenotype analysis of *spl36*

At the ninth-leaf stage, *spl36* began to develop lesion-like brown mimics on the lower leaf sheath extending upwards along the midrib of the leaf sheath to the leaf

blade, and the phenotype continued with the development of the plant until the maturity stage ([Fig. 1A, 1B](#)). Some lesion mimics were also observed on the glumes at the heading stage, and brown mimics covered almost all the leaves and glumes ([Fig. 1C–1F](#)). Thus, the formation of lesion mimics was positively correlated with leaf age in *spl36*. The results of DAB staining showed brown deposits around the lesions on the leaf blades ([Fig. 1G](#)), indicating ROS accumulation in *spl36*. The results of trypan blue staining showed clear results for the lesions on the leaves of mutant *spl36* and the surrounding area ([Fig. 1H](#)).

Agronomic traits of WT and mutant *spl36* plants were investigated at the maturity stage ([Fig. 2](#)). Compared with WT, the plant height, effective panicle number, panicle length, seed number per panicle, seed setting rate, and thousand-grain weight of mutant *spl36* were significantly reduced by 14.76%, 37.76%, 12.62%, 60.28%, 22.36%, and 12.84%, respectively. The results showed that plant growth and development were severely affected.

Observation results of chloroplast autofluorescence

In the maturity stage, the cross-section of the leaf blade in WT and *spl36* plants was observed under natural light; it was green in the WT plant, whereas mutant *spl36* had some brown mesophyll cells ([Fig. 3A, 3B](#)). Under the ultraviolet light, chloroplast autofluorescence showed that the red fluorescence of mutant *spl36* was significantly weaker both at the parts with or without lesions compared to WT ([Fig. 3C, 3D](#)). The results showed that the mesophyll of the *spl36* mutant may suffer from degradation or be destroyed with the development of lesions.

Pigment content and photosynthetic characterization

We determined and analyzed the photosynthetic pigment content and photosynthetic characteristics of the WT and mutant. The results of photosynthetic pigment content determination showed that compared with the WT, the chlorophyll a, chlorophyll b and carotenoid contents of the mutant *spl36* were all reduced, and all reached extremely significant levels ([Fig. 4A–4C](#)). This indicates that mesophyll cell degradation had indeed occurred in the mutant. Analysis of photosynthetic characterization at the heading

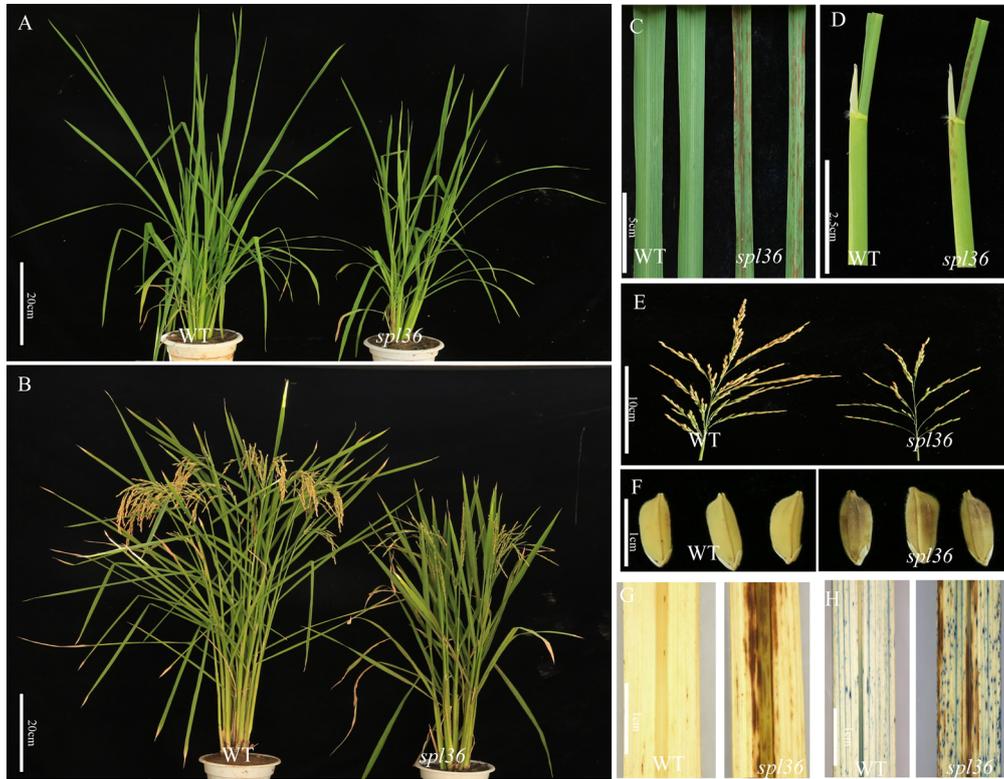


Fig. 1. Phenotype of WT and mutant *spl36* plants. A: WT and mutant *spl36* plants at the tillering stage; B: WT and mutant *spl36* plants at the maturity stage; C: Leaves of WT and mutant *spl36* at the tillering stage; D: Leaf sheath of WT and mutant *spl36* plants at the tillering stage; E–F: Paddy and panicle WT and mutant *spl36* plants at the maturity stage; G: DAB staining of WT and *spl36* leaves; H: Trypan blue staining of WT and *spl36* leaves.

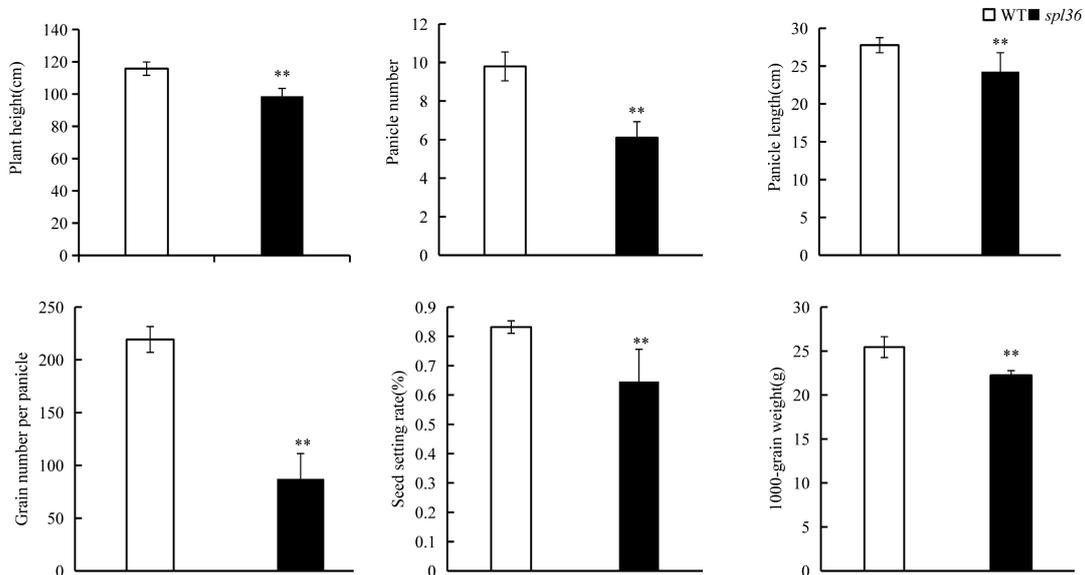


Fig. 2. Agronomic traits of WT and *spl36* mutant plants (** $P < 0.01$, Student's *t*-test).

stage found that compared with the WT, the net photosynthetic rate, stomatal conductance and transpiration rate of the mutant *spl36* leaves were extremely significantly reduced (Fig. 4D, 4E, 4G). At the same time, the inter-

cellular carbon dioxide content of the first leaf blade was reduced extremely significantly, the intercellular carbon dioxide content of the remaining leaves was increased extremely significantly (Fig. 4F).

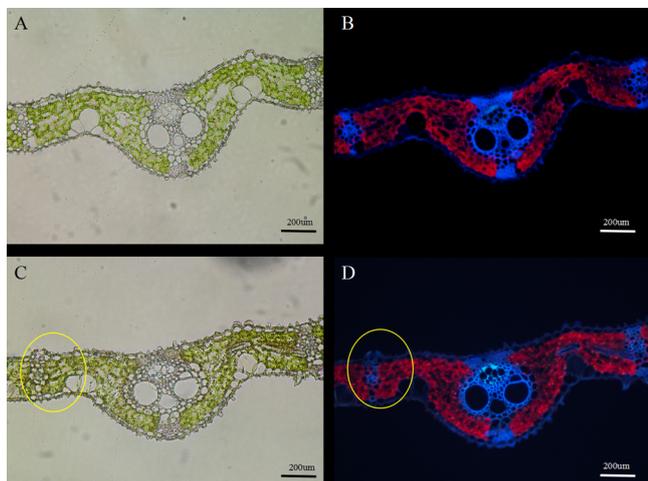


Fig. 3. Fluorescence microscopy of leaf blades of WT and *spl36* mutant plants. A: Schematic cross-section of the WT in a natural light field; B: Cross-cutting diagram of WT under a fluorescent microscope; C: Schematic diagram of cross-cutting of mutant *spl36* under natural light; D: Cross-cutting schematic diagram of mutant *spl36* by fluorescence microscopy; the lesion-forming part is indicated by the yellow circle.

Physiology and biochemistry analyses

To further verify whether generation of the mutant phenotype was accompanied by ROS accumulation, we evaluated the ROS content and activities of antioxidant enzymes in WT and mutant *spl36* at the heading stage (Fig. 5A–5C). The results show that the contents of H₂O₂ and O²⁻ in the mutant were extremely significantly increased compared

with WT, consistent with the results of DAB staining. Thus, the generation of lesion mimics in *spl36* was accompanied by the accumulation of active oxygen, whereas the content of ·OH was lower than that of WT, and this difference reached statistical significance. The enzyme activities of CAT, SOD, POD and other antioxidant in mutants were analyzed in the WT and *spl36* (Fig. 5D–5F). The results show that CAT activity in the first, second, and third leaves was significantly lower in mutant *spl36* than in WT. Meanwhile, POD activity in the first, second, and third youngest leaves was significantly reduced, with the most reduction observed in the last group. Similarly, T-SOD activity was lower in the flag, second, and third leaves in *spl36* than in WT.

Gene mapping of *spl36*

To determine the gene accounting for the phenotype in *spl36*, the F₁ progeny derived from a cross between Xinong1A and the *spl36* mutant showed lesion mimics on the leaves similar to those seen in *spl36*. The F₁ plants exhibited lesions and the F₁ plants were self-crossed to obtain F₂ plants to conduct map-based cloning. In the population containing 2556 F₂ individuals, 721 plants had normal green leaves and 1835 plants had a lesion mimic leaf trait, at a ratio of 3:1 ($\chi^2 = 2.48 < \chi^2_{0.05} = 3.84$). These data indicate that the mutant phenotype is controlled by a pair of dominant nuclear genes. Therefore, we selected F₂ individual plants with the WT phenotype for gene mapping. Ten normal plants from the F₂ plants were selected to construct a normal gene pool. The marker J1216-4 from Chr 11 showed polymorphism, indicating that the target gene

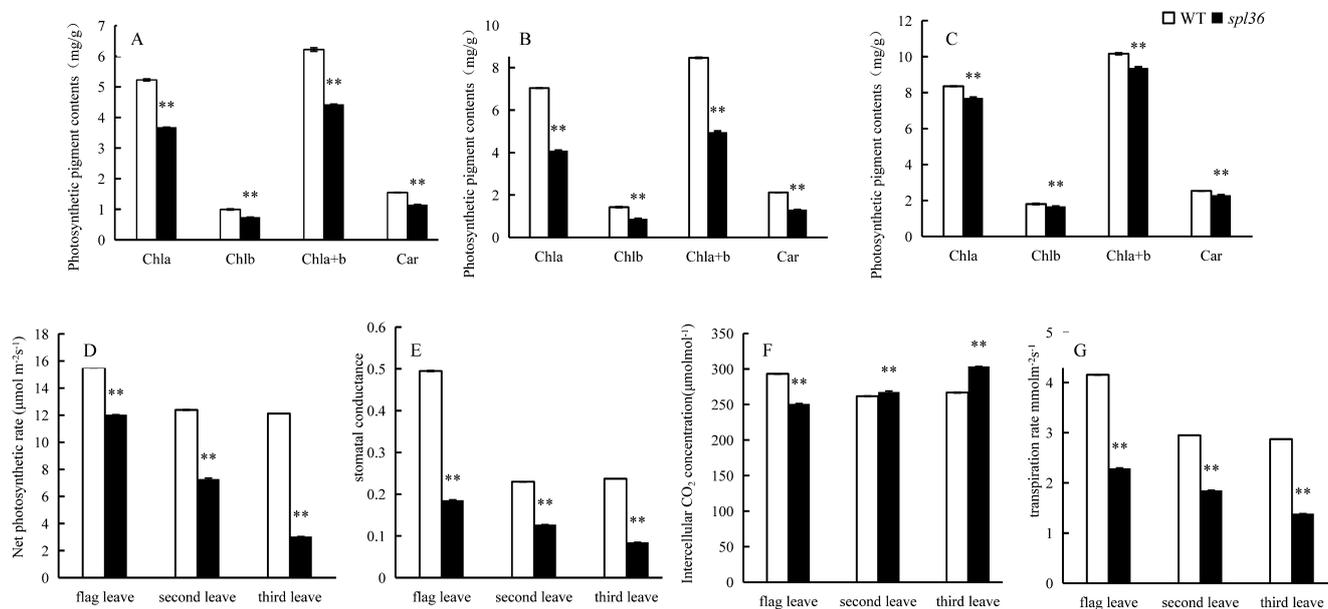


Fig. 4. Photosynthetic pigment content and photosynthetic efficiency of the WT and *spl36* mutant at the tillering stage. A–C: Photosynthetic pigments contents of the flag leaves (A), second leaves (B), third leaves (C) respectively in the wide type and the *spl36* mutant at the heading stage; D–G: net photosynthetic rate (D), stomatal conductance (E), intercellular CO₂ concentration (F), transpiration rate (G) in the WT and the *spl36* mutant at the heading stage. **indicates extremely significant difference between traits ($P < 0.01$, Student's *t*-test).

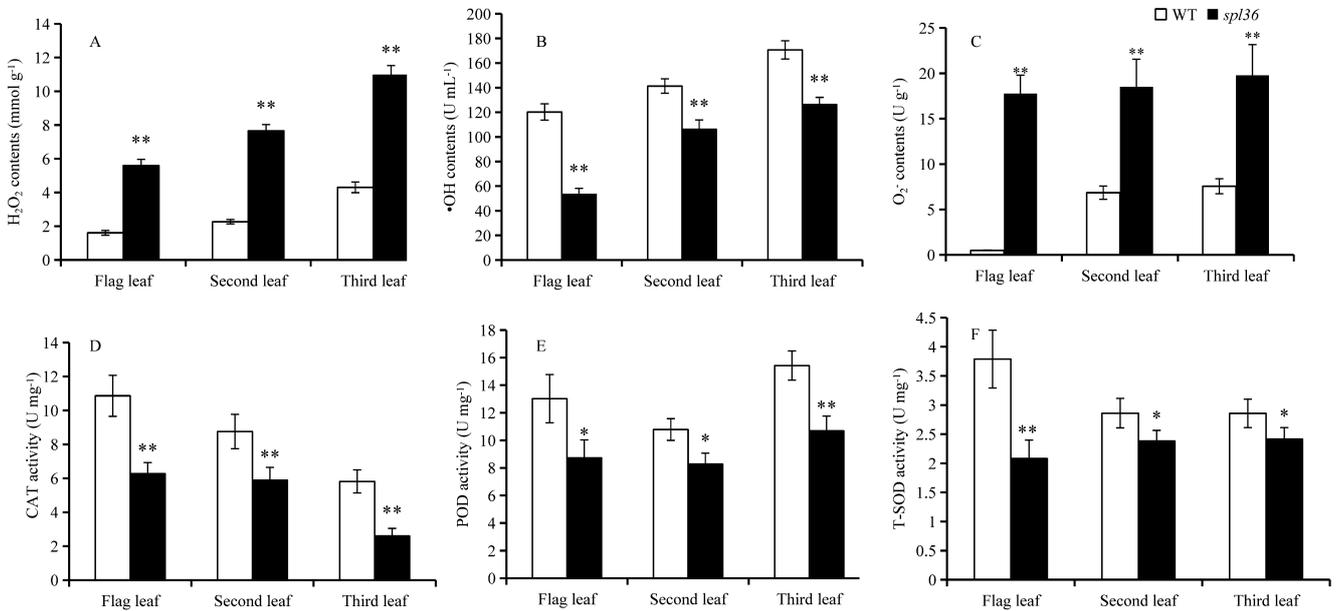


Fig. 5. Physiological index determination of WT and *spl36* mutant at the heading stage. A: Determination of H₂O₂ content; B: Determination of ·OH content; C: Determination of O₂^{·-} content; D: CAT activity; E: POD activity; F: T-SOD activity. * and ** indicate significant difference between traits at $P < 0.05$ and $P < 0.01$, respectively.

might be located on Chr 11 (**Fig. 6A**). To fine-map the gene, more InDel markers were designed, of which nine had a polymorphism. Using genetic linkage analysis, the target gene was further delimited to a 260 kb region between InDel markers J1206-3 and J1206-4 on the long arm of chromosome 11 (**Fig. 6B**).

Expression analysis of PR genes

Lesion mimics often lead to disorders in the plant defence response. To analyze whether *spl36* is involved in the defence response in rice, the expression of disease-related genes was analyzed and the relative expression of defence-related genes *PR1a* (Agrawal *et al.* 2000a), *PR1b* (Agrawal *et al.* 2000b), *PR10* (Kim *et al.* 2004), and *NPR1* (Yuan *et al.* 2007) in WT and mutant plants was measured by qPCR. Before and after the occurrence of lesions in

spl36, the expression levels of the four defence-related genes *PR1a*, *PR1b*, *PR10*, and *NPR1* were significantly higher in the mutant than in WT (**Fig. 7**). In particular, after the appearance of the lesion mimics, expression of the *PR* genes was further significantly increased. These results show that the increased expression levels of resistance-related genes in *spl36* may contribute to its enhanced resistance to pathogens.

Resistance identification

Because *PR* genes are activated in *spl36*, we further tested whether *spl36* enhanced resistance to the main diseases in rice. In the rice blast spectrum test (**Table 2**), 59 effective strains, divided into 6 physiological populations and 19 physiological races were obtained according to varieties identified in China. The resistance frequency of mutant *spl36* was 7.41%, 33.33%, and 15.71% higher in the ZB, ZE, and ZG groups, respectively, than in WT. The disease resistance frequency was 22.23% lower in the ZC group than in WT, whereas the disease resistance frequency in the ZA and ZD physiological groups was the same as that in WT. The larger difference between the ZE and ZC populations may be due to the fact that selection of ZE and ZC is caused by fewer physiological races, and the resistance frequency of the mutant to total population is 5.09% higher than in WT. The broad-spectrum resistance of the mutant to rice blast was still slightly improved (**Table 3**).

At the booting stage, inoculation of *R. solani* was conducted. At 30 days after *R. solani* inoculation, the WT leaf sheaths had all turned yellow and withered, and showed a tendency to spread upwards, whereas the mutant only produced two small water-spot mimics that were not connected

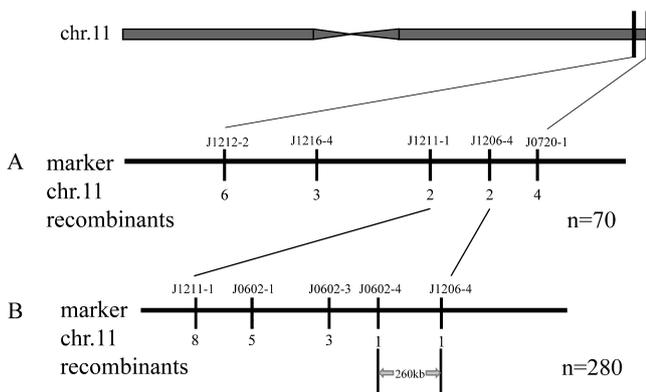


Fig. 6. Mapping of *spl36*. A: Linkage mapping of *spl36*. B: Fine mapping of *spl36*.

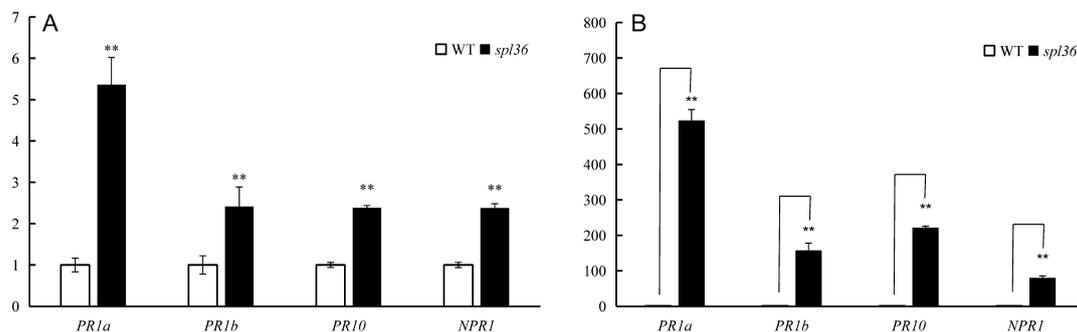


Fig. 7. Expression analysis of disease-related genes. A: The expression of disease-related genes in WT and mutants before lesion mimics phenotype; B: The expression of disease-related genes in WT and mutants after lesion mimics phenotype. **indicates extremely significant difference between traits ($P < 0.01$).

Table 2. Number of effective isolates and physiological races as well as occurrence frequency in the physiological group

No. of effective isolates	No. of races	Occurrence frequency of physiological race group (%)				
		ZA	ZB	ZC	ZD	ZE
59	19	7.14%	45.76%	15.25%	5.08%	5.08%

Table 3. Resistance spectrum determination of the WT and *spl36* mutant

Materials	Resistance frequency to each physiological group (%)						Resistance frequency to total population (%)
	ZA	ZB	ZC	ZD	ZE	ZG	
WT	50%	14.81%	66.67%	66.67%	33.33%	69.23%	40.67%
<i>spl36</i>	50%	22.22%	44.44%	66.67%	66.67%	84.61%	45.76%

(**Fig. 8A**). After 30 days of rice heading, the incidence of sheath blight was stable. Field investigations on the incidence of WT and mutants showed that there were more disease mimics on WT than on mutants, with statistical significance. Because the WT plant is much taller than the mutant, we further investigated the relative height of the WT and mutant lesions, and found that the relative lesion height of the WT was significantly higher than that of the mutant, indicating that the WT disease was more severe than the mutant equivalent (**Fig. 8C**). The disease level was significantly higher in WT than in the mutant, indicating that lesion mimics enhanced the mutant resistance to sheath blight.

At the tillering stage, the resistance to bacterial pathogen *Xoo* was evaluated in the WT and mutant *spl36*. At 21 days after the inoculation of bacterial blight, the lesion lengths of WT and mutant *spl36* bacterial blight were investigated. At the inoculated leaf tips, the leaves turned yellow and withered, and bacterial blight continued to spread (**Fig. 8B**). The length of the lesion was significantly greater than that seen in the mutant *spl36*, indicating that *spl36* participates in *Xoo* resistance in rice (**Fig. 8D**).

Discussion

In the absence of pathogen infection and adversity, LMMs spontaneously form lesions in leaf sheaths or leaf blades

similar to those produced by pathogen infection, which is often accompanied by a growth defect and development and activation or silencing of disease resistance (Qiu *et al.* 2014). The *ttm1* mutant (Takahashi *et al.* 2007) exhibits short growth, spotted leaves, and *OsPti1a* negatively regulates basic resistance to pathogens in rice. The leaves of *OsSSI2* (Jiang *et al.* 2009) insertion mutation and interference plants showed spontaneous damage and slow growth. No difference in the early development stage was observed between the *spl3* (Wang *et al.* 2015b) mutant and WT. At the late tillering stage, disease mimics gradually appeared on the older leaves and black mimics developed on the whole leaves at the heading stage with stunted growth and decreased agronomic characteristics, especially fertility and panicle length.

In this study, we identified a novel LMM *spl36*, which appeared in the leaf sheath of the plant at the 9-leaf stage. As the plant grew and developed, it continued to spread to the leaf blades and glumes, and no lesion mimics were observed on stems or other tissues in the *spl36* mutant. Compared with WT, the plant height, effective panicle number, panicle length, seed number per panicle, seed setting rate and 1000-grain weight of the mutant *spl36* were significantly reduced, showing that the mutation seriously affected the growth and development of mutant *spl36* plants.

The formation of many types of lesions is accompanied

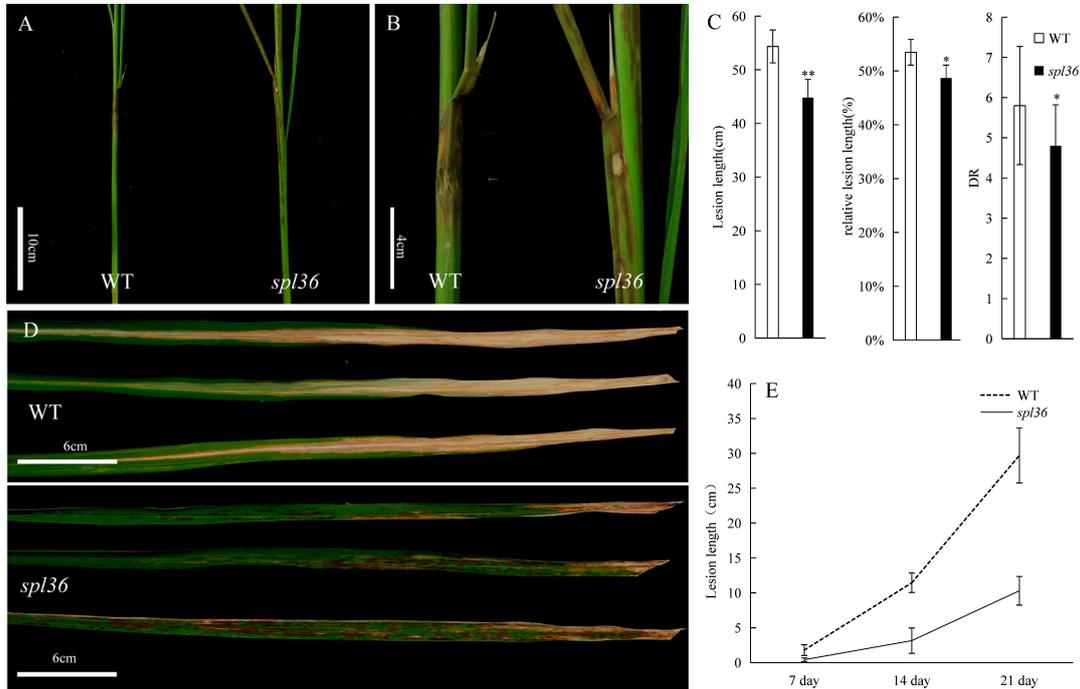


Fig. 8. Identification of resistance to sheath blight and bacterial blight. A: The incidence of wild type (WT) and mutant *spl36* sheath blight; B: Flag leaves of WT and mutant after inoculation; C: Statistics on the incidence of wild-type and mutant sheath blight; D: Statistics on the incidence of wild-type and mutant bacterial blight.

by excessive ROS accumulation. The large accumulation of ROS not only destroys the cell structure, but also functions as a signal molecule to induce the activation of HR to cause PCD, which results in the production of lesion mimics on plant tissues (Matin *et al.* 2010). When treated with fungal inducers, the mutants *spl2*, *spl7*, and *spl11* (Kojo *et al.* 2006) accumulate more H_2O_2 in the cells. In mutant *cea62* (Liu *et al.* 2012), the accumulation of H_2O_2 damaged the cell membrane and caused cell death on the leaves. In our study, the results of trypan blue and DAB staining showed excessive accumulation of H_2O_2 and O_2^- in the mesophyll cells, and the activities of protective enzymes such as CAT, POD, and SOD were reduced, resulting in excessive ROS accumulation in the mutant. Together, these results suggest that the excessive ROS accumulation leads to the PCD of mesophyll cells and the formation of lesion mimics. The much weaker autofluorescence of chloroplasts, decreased photosynthetic pigment content, and reduced net photosynthetic rate, stomatal conductance, and transpiration rate in *spl36* indicate that the degradation of chloroplasts and reduced photosynthesis may account for the senescence of leaf blades and the severe reduction of the main agronomic traits.

Many LMMs show diverse enhanced resistance to the corresponding pathogens in plants, due to the activation and elevated expression of defence response marker genes. In the *PR* family, the *PR1* subfamily is often used as a marker gene for systemic resistance, of which *PR1a* and *PR1b* reportedly play a role in the abiotic stress response

(Agrawal *et al.* 2001). *PR10* induces cell death in rice, Arabidopsis, and tobacco through RNase activity inside the cell (Kim *et al.* 2011). *NPR1* is a positive regulator of cell death in rice (Liu *et al.* 2017). For example, in LMM *OsIsd1*, expression of the defence response marker genes *PR1* and *PR10* was significantly increased with enhanced resistance to rice blast. The *tml* mutant has increased expression of *PR1b* and *PR10* also shows enhanced resistance to rice blast and bacterial blight. In this study, the expression of defence-related genes *PR1a*, *PR1b*, *PR10*, and *NPR1* were significantly increased in *spl36* with the appearance of lesion mimics on leaves, which indicates that *spl36* may function in a disease-resistant signal pathway. A pathogen inoculation study found that *spl36* demonstrated enhanced resistance to rice blast, sheath and bacterial blight, which confirms the participation of *spl36* in the defence response.

We identified a new LMM, *spl36*, and fine mapped it to a 260-kb region on chromosome 11. In interval, there are 6 glycosyl hydrolase, 7 hypothetical protein, 8 expressed protein, 3 zinc finger protein, 2 thaumatin family domain containing protein, 1 SNF7 domain containing protein, 1 trp repressor/replication initiator, 1 selT/selW/selH seleno-protein domain containing protein, 1 pollen signalling protein with adenylyl cyclase activity, 1 glucan endo-1,3-beta-glucosidase precursor, 1 RNA recognition motif containing protein, 1 GRAS family transcription factor domain containing protein, 1 AAA-type ATPase family protein, 1 MYB family transcription factor and 1 alpha-hemolysin; And

glycosyl hydrolase (Tokunaga *et al.* 2008), MYB family transcription factor (Kishi-Kaboshi *et al.* 2018) and zinc finger protein have been reported to play a role in the defense mechanism of rice. In addition, 4 genes have been reported. *MT1A* (Yang *et al.* 2009) encodes type 1 metallothionein. *OsMT1a* can directly participate in the ROS clearance pathway and change the balance of Zn²⁺ in the plant to regulate the expression of zinc finger structure transcription factors to improve plant stress resistance. *CycD7* (La *et al.* 2006) is a rice cyclin gene. *ZOS11-10* (Figueiredo *et al.* 2012) can bind to *OsDREB1B* promoter. *DHD1* (Zhang *et al.* 2019) is a GRAS protein, and *DHD1* is involved in the flowering pathways related to GA and ABA. The promoter, ORF, and 3'UTR sequence of reported genes in this range have been sequenced and no base difference was found. And glycosyl hydrolase (Tokunaga *et al.* 2008), MYB family transcription factor (Kishi-Kaboshi *et al.* 2018) and zinc finger protein have been reported to play a role in the defense mechanism of rice. We speculate that these genes may be target genes. Next, we will expand the F₂ group to narrow the interval. At the same time, We will detect variant sites for wild-type and mutants to determine our target gene for future functional study. Together, we did fine mapping of a non-cloned gene that functions in the spectrum resistance response in rice, the cloning of which will be of great value to science and agriculture.

Author Contribution Statement

ChangWei Zhang conceived and designed the research. LinJun Cai, Meng Yan, Han Yun, Jia Tan, Dan Du, Hang Sun, YunXia Guo, XianChun Sang assisted in the experiments. LinJun Cai and Meng Yan analyzed the experimental data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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