SPL36 encodes a receptor-like protein kinase precursor and regulates programmed cell death and defense responses in rice

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Abstract

The rice (*Oryza sativa*) spotted leaf 36 (*spl36*) mutant was identified from an ethyl methanesulfonate–mutagenized Japonica cultivar Yundao population and was previously shown to display a spontaneous cell death phenotype and enhanced resistance to rice bacterial pathogens. Through the analysis of the expression of related genes, we speculate that *spl36* is involved in the disease response by up-regulating the expression of defense-related genes. The results of physiological and biochemical experiments indicated that more cell death occurred in the mutant *spl36*, and the growth and development of the plant were affected. We have isolated *SPL36* via a map-based cloning strategy. A single base substitution was detected in *spl36*, which results in encoding amino acid changes in the SPL36 protein. The predicted *SPL36* encodes a receptor-like protein kinase precursor that contains repeated leucine domains and may be involved in stress response of rice. In the salt treatment experiment, we found that the mutant *spl36* showed sensitivity to salt. Therefore, *SPL36* may negatively regulate salt stress-related responses.

Background

Lesion mimic is the spontaneous production of disease spots of different sizes and shapes on leaves and leaf sheaths, and even stalks and seeds without both abiotic and biotic stresses. These lesion mimics are the result of apoptosis caused by allergic responses (HR) (Petrov et al., 2015). Lesion mimic mutants of rice can be divided into an initial type and a spread type according to the phenotype and dominant and recessive mutation types, with different mutation types varying in phenotype. The first reported lesion mimic mutant in plants was identified in maize by the American scientist Emerson in the 1920s (Lu et al., 2012). Sekiguchi Lesion (sl) which is the first lesion mimic mutant in rice was discovered by the Japanese scientist Sekiguchi in the mid-1960s as a mutant formed by natural mutation (Liu et al., 2004). *SPL7* is the first lesion mimic mutant gene successfully cloned in rice and encodes the HSFA4 heat shock protein transcription factor which plays a negative role in the apoptosis pathway (Yamanouchi et al., 2002). *SPL7* is highly homologous to maize *HSFb*, tomato *HSF8*, *Arabidopsis HSF21* and *HSF1*, all these mutants regulate apoptosis in plants and show lesion mimic characteristics. The mechanism of lesion mimic generation is complex and regulated by multiple factors, including both internal and external factors. Internal factors include the involvement of disease resistance-related genes, uncontrolled programmed cell death (PCD), metabolic disorders, defense signaling molecules, and loss of protease function; external factors include temperature and light. For example, the phenotype of *spl18* is associated with the insertion of a T-DNA activation tag which enhances the expression of genes around the insertion site (Mori et al., 2002). The *OsATL* gene encoding an acyltransferase homolog that induces allergic reactions in tobacco lies about 500 bp downstream of the inserted T-DNA activation tag. This gene is expressed at a lower level in wild-type rice, but at a high level in *spl18*, resulting in the occurrence of lesion mimics due to abnormal expression of rice disease resistance genes. Mutation of rice *NLS1* that encodes the CC-NB-LRR protein, H_2O_2 and salicylic acid (SA) accumulate in large amounts in the mutant, and the abnormal expression of resistance-related genes leads to the appearance of lesion mimics in the rice leaf sheaths (Tang et al., 2011). Zeng found that the *spl11* protein contained U-box and ARM

(armadillo) repeat domains, and might undergo ubiquitination and protein-protein interactions in yeast and mammalian systems (Zeng et al., 2002). Finally, the comparison of amino acid sequences showed that the similarity of *spl11* with other plant U-box-ARM proteins was mainly limited to the U-box and ARM repeat regions, and a single base substitution was detected in the *spl11* mutant gene, which resulted in the premature termination of translation of *spl11* proteins. In addition, in vitro ubiquitination assays showed that the *spl11* protein had E3 ubiquitin ligase activity which was dependent on the intact U-box domain, indicating that ubiquitination plays a role in plant cell death and defense, which further suggests that spontaneously formed lesion mimics are associated with uncontrolled PCD (Zeng et al., 2004). Moreover, *OsSSI2* encoded fatty acid dehydrogenase (FAD), which also plays a negative role in the rice defense response, resulting in lesion mimics and delayed growth of rice leaves after loss of FAD function (Jiang et al., 2009). Furthermore, mutations in uridine diphosphate-N-acetylglucosamine pyrophosphorylase (UAP1) during glucose metabolism can also lead to the appearance of lesion mimics in rice leaves (Jung et al., 2005). According to the current study, most rice lesion mimic mutants show enhanced resistance to some extent. Among the more than 80 mutants that have been identified, 11 mutants such as *spl1*, *spl9*, *spl10*, *cdr1*, and *cdr3* showed enhanced blast resistance(Liu et al., 2004; Yoshimura et al., 1997; Takahashi et al., 1999); 12 mutants such as *spl21*, *spl24*, *lmes1*, *hm197*, and *hm83* showed enhanced bacterial blight resistance(Wu et al., 2008); 19 mutants such as *spl14*, *bl3*, and *Lmr* showed enhanced blast resistance and bacterial blight resistance(Mizobuchi et al., 2002); and mutant *lmm1* showed both enhanced blast resistance and sheath blight resistance; among them, mutants *spl2*, *spl3*, *spl4*, *spl6*, *spl7*, and *ncr1* showed no enhanced resistance, and their resistance was unchanged or even reduced(Kang et al., 2007; Campbell et al., 2005) .

Plant receptor-like protein kinases (RLKs) occupy important metabolic positions and are abundant in plants, with about 1130 RLK genes in rice (Nguyen et al., 2015). RLKs in plants are structurally similar to PRKs in animals and are composed of intracellular, extracellular, and transmembrane regions (Ye et al., 2017); Most RLKs have an extracellular receptor domain (ECLB), a transmembrane domain (TM) and a protein kinase contact response domain (PKC) (Walker, 1994; Zhang, 1998). The leucine-rich repeats (LRRs) class of receptor-like protein kinases are a subtype of receptor-like protein kinases. Plants are continuously subjected to biotic and abiotic stresses such as cold, heat, drought, waterlogging, salt, and pests. LRR-type receptor protein kinases are involved in plant stress responses and defense-related processes, and LRR-type receptor protein kinases related to plant disease resistance have drawn great attention. It has been reported that the extracellular domains of proteins encoded by the *Cf* gene family of tomato leaf mold have LRR structures, and that differences in the amino acid sequences of the LRR motifs of different proteins in the same family are responsible for their specificity of ligand binding (Thomas et al., 1998). The resistance gene *FLS2* of *Arabidopsis* has a similar structure to the extracellular domain of the tomato *Cf* gene family (Gómez-Gómez et al., 2001). The extracellular LRR structure of rice *Xa21*, on binding to ligands (avirulent gene products of rice bacterial blight pathogens), can induce intracellular kinase phosphorylation and produce a series of cellular responses that protect the rice from pathogens (Song et al., 1995; Park and Ronald, 2012). These findings indicate that the LRR structure plays an important role when it binds to pathogens. In addition, Lee et al(Lee et al., 2004) found that the

LRR type receptor protein kinase *OsRLK1* gene of rice could be induced by low temperature and salt stress, and Junga et al(Junga et al., 2004) also found that expression of *CALRR1* of pepper could be induced not only by anthrax pathogens but also under abiotic stress conditions such as high salt, abscisic acid (ABA), and wounding. To further explore the signal transduction pathways of LRR-type receptor kinases in response to stress signals, we isolated and characterized a novel rice lesion mimic mutant, *spotted leaf* 36 (*spl36*). This mutant shows spots at the tillering stage and enhanced resistance to bacterial blight. We cloned the *SPL36* gene by map-based cloning and demonstrated that it encodes a receptor-like protein kinase receptor that is expressed in all tissues and developmental stages and encodes the protein *SPL36* located at the plasma membrane. A high degree of cell death, changes in chloroplast structure, and activation of defense-related responses were observed in *spl36* mutants. The experimental results indicate that the loss of *SPL36* function is responsible for cell death regulation, premature senescence, and defense response activation.

Materials And Methods

Plant materials and growth conditions

The spotted leaf mutation *spl36* was isolated from a methanesulfonate (EMS)-induced mutant library of Yundao rice (wild-type, WT). Hybridization was performed with TN1 as the male parent and mutant, and the F_1 offspring and F_2 population were grown in the rice experimental field of Zhejiang Normal University, Jinhua City, Zhejiang Province, China, during the summer of 2018 and 2019. The F_2 population of the *spl36*/ZF802 cross was used for genetic analysis and the F_2 population of *spl36*/TN1 was used for map-based cloning. The agronomic traits of wild-type and mutant *spl36* were also statistically analyzed, including plant height, tiller number, grain number per panicle, seed setting rate, and 1000-grain weight. The results were analyzed using the average of 10 replicates.

Determination of Photosynthetic Parameters and Chlorophyll Content

From 9:30 a.m.to 11:00 a.m. on sunny days, 10 individual plants with relatively uniform growth were harvested. The photosynthetic parameters of the wild-type and mutant were measured by the LI-6400XT portable photosynthesis tester. Three to five representative flag leaves were treated and measured, and each leaf was measured in triplicate (the mean value was taken as one replicate). During the measurement, red and blue light sources were used, the light intensity was constant at 1200 μmol/m^2, the temperature was 30 °C, the CO_2 concentration was the concentration in the air, and the humidity was the humidity in the atmosphere. Five wild-type and mutant plants with relatively uniform growth vigor were selected. 0.05 g of the leaves were taken after weighing and then soaked in 25 mL 1:1 ethanol: acetone solution after being cut into pieces; three duplications were set and subject to the darkening reaction for 24 hours, followed by shaking. The absorbance values at 663 nm, 645 nm, and 470 nm were measured

with a spectrophotometer, and the photosynthetic pigment content was calculated and statistically analyzed by the t-test.

Histochemical Analysis

The content and concentration of malondialdehyde (MDA), as well as the enzymatic activity and superoxide dismutase of peroxidase (POD) were compliant with the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The contents of MDA and H_2O_2 as well as the enzymatic activities of SOD and POD were all measured when the phenotype of the tiller stage mutant *spl36* had just appeared. Apoptosis was detected by the TUNEL method, FAA fixative was prepared before sampling, and fixative was added to a 2 mL centrifuge tube (Liang et al., 2018). At the stage when the lesion mutant phenotype was apparent in the mutant *spl36* leaves, the leaf tissues showing the phenotype were harvested and the leaves at the corresponding position of the wild-type were harvested, cut into clumps and placed in the 2/3 position of FAA fixative for fixation. They were then vacuumed until the samples sank to the bottom, sealed with parafilm and stored in a refrigerator at 4 °C. The TUNEL apoptosis detection kit (Roche, Cat No.1684817) was used to determine apoptosis in the samples (Inada et al., 1998).

Linkage analysis and mapping of spl36

SSR primers evenly distributed over the 12 chromosomes of rice stored in our laboratory were used to screen the mutants and TN1 for polymorphisms (Supplementary Table 2). Twenty-one F_2 lesion mimic phenotype single plants of *spl36*/TN1 were used for linkage analysis to preliminarily confirm the chromosomal location of the target gene. A new InDel marker with a relatively good polymorphism was further developed in the mapping interval, and the target gene was precisely mapped with a single plant showing the mutant phenotype in the F_2 segregating population of *spl36*/TN1. Genomic DNA was extracted using the hexadecyltrimethylammonium bromide (CTAB) method (Wu et al., 1993). The 10 μL PCR system included: DNA template 1 μL, 10 × PCR buffer 1 μL, forward and reverse primers (10 μmol/L) 0.5 μL each, dNTPs 1 μL, rTaq 0.2 μL, with the addition of H_2O to make up to 10 μL. The PCR amplification program was as follows: pre-denaturation at 94 °C for 4 min; denaturation at 94 °C for 30 s, annealing at 55 °C– 60 °C for 30 s (temperature varied according to primers), extension at 72 °C for 30 s, 40 cycles; and finally extension at 72 °C for 10 min. PCR products were electrophoresed on a 4% agarose gel, and then photographed and stored in a gel imager and the data were read. Primers used for mapping (Supplementary Table 3).

Vector Construction

For functional complementation of rice *spl36* mutants, the complete genomic DNA fragment including the promoter of *SPL36* in the wild-type was amplified by PCR with primers spl36-CPT-F/36-CPT-R, and then the constructed transformant was generated by inserting the empty binary vector pCAMBIA1300 through Clontech In-Fusion PCR (TaKaRa). The full-length *SPL36* open reading frame (ORF) was amplified with the primer pair spl36-GFP-F/spl36-GFP-R, and the coding sequence (CDS) of *SPL36* was

inserted into the binary vector pHQSN containing the 35S promoter (p35S: SPL36) for sublocalization of cells. The *SPL36* promoter was constructed into the expression vector pCAMBIA1305.1, and the expression of *SPL36* in rice tissues was revealed by using GUS reporter gene. CRISPR Cas9-gRNA vector was constructed by Jiangsu Baig Gene Technology Co., Ltd. All binary constructs were introduced into the corresponding generated wounded tissues by *Agrobacterium tumefaciens* (EHA105)-mediated method for validation of subsequent assays (see Supplementary Table 4 for required primers). The fluorescence of GFP was observed by confocal laser scanning microscopy (Leica TCS SP5, Leica, Germany), and the primers used for vector construction are shown in Supplementary Table 4.

Inoculation Test

The Xanthomonas oryzae pv. oryzae (causal agent for bacterial blight) was inoculated onto the flag leaves of wild-type Yundao and mutant *spl36* at the tillering stage. After inoculation, the phenotype of the inoculated leaves was observed at 5 and 10 days and the lesion length were measured and photographed.

Quantitative Real-Time PCR Analysis

Leaf, root, stem, leaf sheath, panicle, and grain samples of wild-type and mutants at each stage were taken and analyzed using RNAprepPure Plant Kit (Cat No. DP441, Tiangen Biotech, Beijing, China) to extract RNA, according to the instructions. The extracted RNA was amplified using a ReverTra-Plus-reverse transcription kit (Cat No.FSQ-301, Toyobo, Japan) for post-reverse transcription backup. Real-time PCR (qRT-PCR) was used to detect the expression of defense-related genes and the expression of *SPL36* in tissues at each stage, with the OsActin gene used as an internal reference (GenBank accession number: NM001058705). Reaction system: 2 μL cDNA template, 10 μL 2 × SYBR qPCR mix, forward and reverse primers 0.8 μL each, with the addition of ddH_2O to make up to 20 μL. The reaction program was 95 °C for 30 seconds; 95 °C for 5 seconds, 55 °C for 10 seconds; and 72 °C for 5 seconds for 40 cycles. Each reaction was performed in triplicate, and the relative expression of premature senescence-associated genes was calculated based on $2^{-\Delta\Delta}$Ct. The real-time PCR instrument was the quantitative fluorescence gene amplification instrument qTOWER3G (Jena, Germany). Data were analyzed by PSS19.0 software and Excel. The t-test was used for significance analysis of differences, and the primers used for qRT-PCR are shown in Supplementary Table 5.

Salt Stress Assay

Plate test: Seeds of full wild-type Yundao and mutant spl36 were selected, washed, spread on 200 mM NaCl MS medium, and cultured at 28 °C under light. In addition, MS medium without NaCl was used as a control. The assay was performed in triplicate, and the germination rate of seeds was observed and counted at each stage. After nine days, root lengths were counted and photographed.

Salt stress assay at seedling stage: The hydroponic wild-type and mutant seedlings were used for about two weeks, and seedlings with approximately the same growth momentum were selected for the assay. Wild-type seedlings and mutant seedlings were transferred to normal nutrient solution and nutrient

solution containing 150 mM NaCl for culture, and four days thereafter, salt-stressed seedlings were transferred to normal nutrient solution for recovery culture for three days to observe plant survival rate and determine fresh weight, conductivity, and proline content.

Results

Phenotype of spl36 lesion mimic

Under normal planting conditions in summer, the leaves of *spl36* did not change significantly from those of the wild type (WT) before the tillering stage. At tillering stage, the lesion mimic appeared in the leaf apex (Fig. 1A). From the tillering stage to the heading stage, these necrotic spots became more severe and gradually spread to the whole leaf (Fig. 1B). To investigate whether *spl36* is induced by light like most lesion mimics, mutant *spl36* leaves were covered with 2–3 cm aluminum foil at the tillering stage, with the uncovered mutant leaves used as additional controls. After even days, it was observed that no spread of lesion mimics had occurred in the covered area of the covered leaves, while the lesion mimics on the uncovered control leaves (Fig. 1C). This shows that the lesion mimic phenotype arising from mutant *spl36* is induced by light. Meanwhile, the main agronomic traits of mutant *spl36* such as plant height, grain number per panicle, and 1000-grain weight were significantly reduced (Fig. 1D–I).

SPL36 Gene Regulates Plant Growth and Development

Because of the negative agronomic changes in mutant *spl36*, and chloroplasts are the main site of photosynthesis. We speculated that the growth and development of the plants were affected after the appearance of the mutant lesion mimic phenotype (Han et al., 2015). We used a transmission electron microscope to observe chloroplast ultrastructure and found that the chloroplasts of mutant *spl36* were atrophied and the volume of chloroplasts became smaller, along with disorganized lamellae inside the chloroplasts (Fig. 2A–D).We speculated that the growth and development of the plants were affected after the appearance of the mutant lesion mimic phenotype. Measurement of the chlorophyll content of wild-type Yundao and mutant *spl36* at the tiller peak revealed that both chlorophyll a and chlorophyll b of mutant *spl36* were significantly reduced compared with the wild-type (Fig. 2E). We further measured the photosynthetic rate of the plants during this period, and the results showed that the net photosynthetic rate of the mutants was significantly reduced (Fig. 2F). Therefore, the *SPL36* gene regulates plant growth and development through changes in chloroplast structure.

SPL36 Regulates ROS Accumulation and Cell Death in Rice

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is designed to detect DNA fragmentation, which is a marker of programmed cell death (Kim et al., 2009). The TUNEL signal in the nuclei of mutant *spl36* cells was intense and randomly distributed, whereas only a weak TUNEL signal was detected in the wild type (Fig. 3A–D). In addition, the accumulation of reactive oxygen species (ROS) at high concentrations leads to an oxidative burst, which causes cell damage and even triggers programmed cell death (Kim et al., 2010). The content of H_2O_2 and the activity of peroxidase (POD) are

directly related to the accumulation of ROS. Superoxide dismutase (SOD) plays an important role in scavenging O^{2-} in plants. Through the detection of H_2O_2 content, POD activity and SOD activity, it was found that a large amount of H_2O_2 accumulated in the mutant *spl36* (Fig. 3E), while the activities of POD and SOD in the mutant *spl36* were significantly reduced (Fig. 3G–H). This decrease in enzyme activity would negatively affect the removal of the related peroxide and negative oxygen ions, resulting in the accumulation of ROS. In addition, membrane lipid peroxidation occurs when plant organs age or suffer damage under stress. Malondialdehyde (MDA) is the final decomposition product of membrane lipid peroxidation, and the content of MDA can reflect the degree of damage in a stressed plant. We found that the MDA content was significantly higher in the mutant *spl36* than in the wild-type (Fig. 3F). These results indicate that the lesion mimics in *spl36* mutants are caused by ROS accumulation and irreversible membrane damage. In addition, loss of *SPL36* function triggers the PCD pathway, ultimately leading to the appearance of the *spl36* lesion mimic phenotype.

SPL36 Regulates Defense Responses in Rice

It has been reported that most rice lesion mimic mutants have enhanced resistance to pathogens. To investigate whether the resistance of mutant *spl36* to rice pathogens was enhanced, we performed an inoculation assay on wild-type Yundao and mutant *spl36* at the tillering stage, and used the leaf clipping method to plant the rice bacterial blight strain HM73. Changes in the inoculation site and the length of the lesion mimics were observed at 5 and 10 days after inoculation, respectively. We found that the leaf apex of the wild-type showed obvious necrotic spots at five days after inoculation, while the mutant did not show obvious disease spots; the length of the wild-type disease spots was significantly longer than that of the mutant 10 days after inoculation (Fig. 4A–E). This shows that the resistance to bacterial pathogens is significantly enhanced after the emergence of the mutant *spl36* disease spots. To further explore the mechanism of enhanced resistance of mutant *spl36* to bacterial pathogens, we examined the expression of defense-related genes in wild-type and mutants at the tillering stage by using qRT-PCR, and the results showed that the expression levels of defense genes *MAPK12*, *WRKY53*, *BIMK2*, *AOS2*, *ASP90*, *LYP6*, *PR2*, *PR1a*, and *PR1b* were significantly elevated (Fig. 4F). Thus, loss of *SPL36*-encoded protein function triggers a rice defense response, which leads to enhanced resistance of mutant *spl36* to pathogens.

Genetic Analysis and Map-Based Cloning of SPL36 Gene

Mutant *spl36* was used as the female parent to be hybridized with ZF802 of the Japonica cultivar TN1. The F_1 plants did not show the phenotype of lesion mimic, and the segregation ratio of normal phenotype and lesion mimic phenotype in the F_2 population was essentially in compliance with the 3: 1 ratio, indicating that the *spl36* phenotype is caused by mutations in a single recessive nuclear gene (Supplementary Table 1). A selection of polymorphic markers from 238 insertion and deletion tags mapped 21 F_2 individuals with a lesion mimic phenotype, and we mapped the mutation site to a location between chromosome 12 B12-5 and B12-6 (Fig. 5A). The *SPL36* location was further refined to a location between JHL-3 and JHL-7 by genotyping 148 mutant F2 individuals from the same cross and adding four

additional polymorphic tags (Fig. 5B). Using an additional 554 F_2 mutant individuals and four newly developed polymorphic tags, we finally mapped *SPL36* to a 60 kb region between markers InDel1 and InDel2 (Fig. 5C). Website inquiry (http://rice.plantbiology.msu.edu/) predicted that the region had 11 open reading frames (ORFs), which included seven expressed proteins and four functional proteins (Fig. 5D). Through sequencing and alignment, we found that the gene *LOC_Os12g08180* was mutated (Fig. 5E), and nucleotide C at position 1462 in the coding region of this gene was replaced with T (Fig. 5F), resulting in the change of the encoded amino acid from arginine to cysteine (Fig. 5G), so *LOC_Os12g08180* was used as a candidate gene for *SPL36*.

Functional complementation of the spl36 **mutant with** LOC_Os12g08180

To verify whether the single base substitution in *LOC_Os12g08180* was associated with the *spl36* phenotype, we constructed the vector *pGSPL36*, which contained genomic DNA fragments including the promoter of the *SPL36* gene in wild-type Yundao, and then introduced it into *spl36* by Agrobacterium tumefaciens-mediated transformation. The corresponding empty vector pEmV was also transformed as a control. Of the 60 T0 plants which had been transformed, 54 were positive transformants, all of which showed the same normal phenotype as the wild-type (Fig. 6A), while the plants transformed with the control vector showed the same lesion mimic phenotype as the mutant *spl36* (Fig. 6B), demonstrating that *LOC_Os12g08180* was *SPL36*, and that the single base substitution in *spl36* led to the appearance of the lesion mimic phenotype of the plants.

Expression pattern analysis of SPL36

We used real-time quantitative PCR (qRT-PCR) to analyze the expression of *SPL36* in various organs. The results showed that *SPL36* was expressed in the organs, with higher expression in leaves, leaf sheaths, and roots and lower expression in stems and panicles. *SPL36* expression was significantly higher in all organs of mutant *spl36* compared to the wild-type Yundao (Fig. 7A). To analyze the spatiotemporal expression pattern of *SPL36* more precisely we constructed the vector p*SPL36*: GUS by fusing the GUS gene with the promoter of *SPL36* in the wild-type. We also utilized *Agrobacterium tumefaciens*-mediated transformation to obtain transgenic plants. We stained various organs of the transgenic positive plants and observed GUS signal maps in various tissues (Fig. 7B–F), which was consistent with the qRT-PCR results. These results suggested that *SPL36* was expressed in all organs and at all developmental stages.

Subcellular Localization of SPL36 Protein

To determine the subcellular localization of *SPL36*, the full-length coding sequence of *SPL36* was fused to the N-terminus of green fluorescent protein (GFP). When transiently expressed in rice protoplasts, the GFP signal appeared on the plasma membrane (Fig. 8A–D). To verify this observation, we transformed the plasmid containing the *SPL36*-GFP fusion vector into *Nicotiana benthamiana* leaves, resulting in the detection of the *SPL36*-GFP protein on the membrane (Fig. 8E–H). These results show that the *SPL36* protein localizes to the membrane.

SPL36 **is involved in salt stress-responsive responses in rice**

After verifying that the single base substitution of *LOC_Os12g08180* was responsible for the lesion mimic phenotype of mutant *spl36*, we found that this gene encodes a receptor-like protein kinase 2 precursor. Plant receptor-like protein kinases play an important role in the process of plant signal transduction and are indispensable carriers which can perceive the signals of growth and development and external environmental stresses by phosphorylation of functional proteins resulting in conformational changes (Lally et al., 2001); plant receptor-like protein kinases also play a regulatory role in plant growth and development and disease resistance defense responses (Afzal et al., 2008; Li Liyun et al., 2008) and most receptor-like protein kinases are related to stress responses. To investigate whether *SPL36* is involved in stress response-related pathways, we performed a salt stress assay in flat dishes and hydroponic seedlings for the wild-type and mutant In the plate assay, in the absence of salt treatment, we found no significant difference in the germination rate of mutant *spl36* and wild-type over a one-week period. In the case of salt treatment, the germination rate of both mutant and wild-type decreased significantly, while the germination rate of the wild-type was also significantly lower than that of the mutant. At day 9 of germination we counted the length of the supra-root portion of the salt-treated and control seedlings, and there was no significant difference in the length of the supra-root portion between wild-type and mutant in the control group while the length of the supra-root portion of the mutant was significantly lower than that of wild-type in the case of salt treatment (Supplementary Fig. 1). In addition, we also treated the wild-type and mutant seedlings hydroponically for four weeks with salt, returning them to normal conditions after three days of treatment. The results showed insignificant changes in the wild-type after three days of treatment with the phenotype recovering after restoration of normal conditions, while the mutant *spl36* showed significant leaf bending after the salt treatment while the phenotype did not recover or even died after restoring normal conditions. Our statistical analysis of fresh weight, conductivity as well as final survival of plants before and after treatment as well as controls revealed that mutant *spl36* was more sensitive to salt treatment (Fig. 9). In summary, *SPL36* is involved in salt stress-responsive responses in rice.

Discussion

Lesion mimic mutants are extremely important in the study of programmed cell death and defense-related responses in plant cells. In the present study, we selected a lesion mimic mutant *spl36* from a mutant library by mutagenizing wild-type Yundao using EMS. There was no obvious phenotypic difference between this mutant and the wild-type at the seedling stage, and reddish-brown disease spots appeared initially at the leaf apex at the tillering stage and then gradually spread throughout the leaf. We observed the chloroplast ultrastructure of both wild-type and mutant at this stage and measured their photosynthetic rate. The results showed that the appearance of lesion mimics led to significant changes in chloroplast structure, as chloroplasts are the main site of plant photosynthesis (Wu et al., 2018), the appearance of lesion mimics affected both the growth and development of the plants. This is also the direct cause of the decline in multiple agronomic traits in mutant plants (Ishikawa et al., 2001). By map-based cloning we mapped the genes within an interval of 60 Kb, according to the data information of the

rice genome database (http://rice.plantbiology.msu.edu/), we found a total of 11 open reading frames (ORFs) within this interval. Seven expressed proteins and four functional proteins were located in this region. The genomic sequences in the mutant and wild-type were amplified by PCR. By sequencing alignment and sequencing analysis, we found that nucleotide Cat position 1462 in the coding region of gene *LOC_Os12g08180* was replaced with T, resulting in the change of the encoded amino acid from arginine to cysteine. Through a functional complementation assay, we determined that this gene was *SPL36*. Structural analysis of the protein encoded by this gene showed that *SPL36* encodes a receptor-like protein kinase receptor containing multiple leucine-repeat domains (Supplementary Fig. 2). Previous studies have shown that leucine-rich (LRR) type receptor protein kinase (LRK) is closely related to the plant stress and defense responses. The *PRK1* gene was isolated from *Arabidopsis* in 1997 by Hong et al(Hong et al., 1997) and the LRR domain and protein-protein interactions of this gene were related to the interaction, but also to the stress signals that perceived the environment; In 2014, Yang et al(Yang et al., 2014)screened new LRR-RLKs in wild soybeans: *GsLRPK*, and confirmed that this could improve drought resistance in *Arabidopsis*; *OsGIRL1* showed an up-regulation response when exposed to abiotic stress-induced salt, osmosis, heat, salicylic acid (SA), and abscisic acid (ABA), but a down-regulation response to jasmonic acid (JA) treatment, and the protein localized to the plasma membrane. The biological function of *OsGIRL1* was investigated by studying the overexpression of genes during irradiation, salt pressure, osmotic pressure, and thermal stress in *Arabidopsis* plants (Park et al., 2014). We found, using a germination assay of flat dish salt stress treatment and salt treatment assay of hydroponic seedlings, that the mutant *spl36* was more sensitive to salt treatment, which may be explained by the fact that a missense mutation of gene *LOC_Os12g08180* in the coding region led to the loss of protein function, the specific mechanism of which remains to be elucidated and which is the main direction of our future study. In previous studies, it has been observed that leucine-rich receptor protein kinases (LRR-LRKs) are mainly associated with abiotic stress responses in plants, while the relationships with PCD and disease resistance have not been reported. We verified a higher degree of cell death in the mutant *spl36* by the TUNEL assay, and further measured the levels of H_2O_2 and MDA and the activities of POD and SOD in the wild-type and mutant; the results showed that mutant *spl36* accumulated more ROS which led to an oxidative burst and ultimately to PCD. Since lesion mimics of *spl36* arise spontaneously, we conclude that *SPL36* negatively regulates PCD in rice. In addition, most of the reported lesion mimic mutants showed some disease resistance, and to verify whether *SPL36* was involved in the disease resistance response in rice, we inoculated the wild-type and mutant with the bacterial blight pathogen HM73 by the shearing method. and found that the mutant *spl36* had significant resistance to this pathogen. However, it remains to be determined whether *spl36* has broad-spectrum resistance as HM73 is only a bacterial pathogen. At the same time, we analyzed the differences in expression of some defense-related genes in the wild-type and mutants, and the results showed that the expression levels of the defense genes *MAPK12*, *WRKY53*, *BIMK2*, *AOS2*, *LYP6*, *PR1a*, and *PR1b* were significantly elevated. *OsWRKY53* is a transcriptional activator that plays an important role in the excitation-induced defense signal transduction pathway in rice (Chujo et al., 2007; Tian et al., 2017). *OsAOS2* expression in leaves is significantly induced by rice blast and driving *OsAOS2* with the PBZ1 promoter activates the expression of other pathogenesis-related genes, thereby increasing the resistance to rice blast (Mei et al., 2006) while

OsBIMK2 plays an important role in rice disease resistance responses (Song et al., 2006). *LYP6*, a protein containing cytolytic enzyme motifs, is a pattern recognition receptor for bacterial peptidoglycan (PGN) and fungal chitin and has a dual role in the recognition of peptidoglycan and chitin in rice innate immunity (Liu et al., 2012). *OsPR1a* and *OsPR1b* are pathogenesis-related genes (Agrawal et al., 2000). Therefore, we hypothesize that *SPL36* regulates the disease resistance response in rice by up-regulating the expression of defense genes, but the specific mechanism needs to be further investigated.

Conclusion

We have cloned a novel spotted leaf gene (*spl36*) in this research,which encodes a receptor-like protein kinase precursor that contains repeated leucine domains and may be involved in stress response of rice. This is the first report of the involvement of a receptor-like protein kinase in rice disease resistance-related pathways. We have shown that loss of *SPL36* function results in enhanced resistance of the mutant to pathogens while enhancing the salt sensitivity of the mutant. Our research is currently conducting an in-depth study on whether the mutant *spl36* has broad-spectrum resistance to pathogens and the involvement of *SPL36* in the mechanism of the salt stress response in rice.

Acronym

Abbreviation	Full name
EMS	ethyl methyl sulfonate
GFP	green fluorescent protein
GUS	β-glucuronidase
LRR-LRK	Leucine repeat Receptor-like protein kinase
PCR	Polymerase Chain Reaction
PCD	Programmed Cell Death
POD	peroxidase
qRT-PCR	Real-time polymerase chain reaction
QTL	quantitative trait locus
RNA	Ribounuceleic acid
ROS	Reactive Oxygen Species
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TUNEL	terminal -deoxynucleotidyl transferase mediated nick end labeling

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article and its additional files.

Competing interests

Not applicable.

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Authors' Contributions

Y. C., W. Y. X., planned and designed the research; J. R., W. X. M., W. S., H. J., Y. H. F. and L. H., performed the experiments; R. Y. C., and J. R. wrote the manuscript; R. Y. C., L. S. F., and R. D. Y. analyzed the data and edited the article. All authors read and approved the final article.

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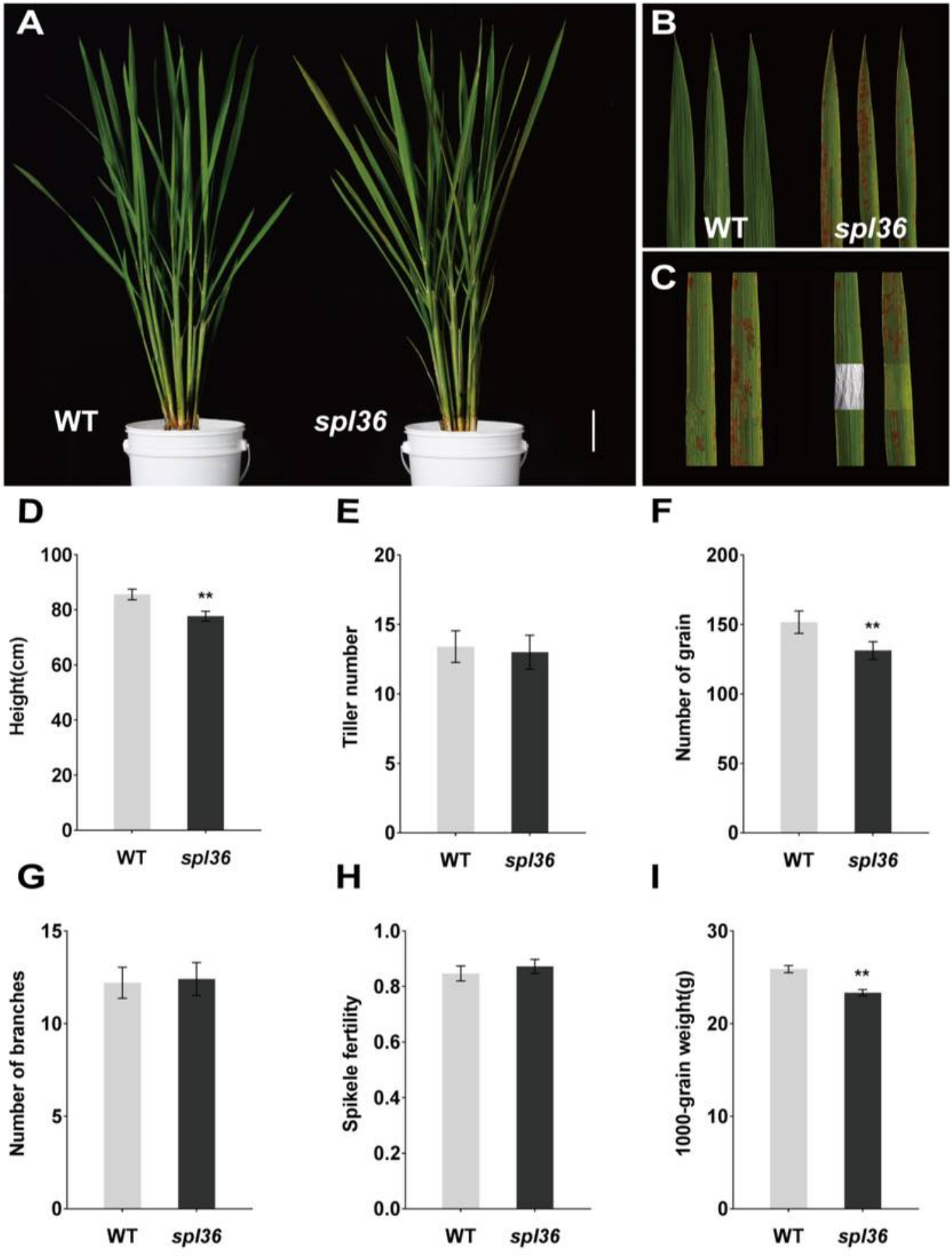
Figures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WT
spl36
B
WT
spl36
C
D
Height(cm)
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80
60
40
20
0
**
WT
spl36
E
Tiller number
20
15
10
5
0
WT
spl36
F
Number of grain
200
150
100
50
0
**
WT
spl36
G
Number of branches
15
10
5
0
WT
spl36
H
Spikele fertility
1.0
0.8
0.6
0.4
0.2
0.0
WT
spl36
I
1000-grain weight(g)
30
20
10
0
**
WT
spl36

Figure 1

Spotted leaf phenotype of spl36 A Lesions appear at tillering stage (bar=6cm). B Lesions appear from the tip of the leaf (WT and spl36 at tillering stage). C Effect of light on lesion formation under the natural condition spl36 before shading (1,3). spl36 shaded for 7 days (2,4). D-I WT and spl36 statistics of important agronomic traits at maturity stage. Values are means ±SD (n=10); ** indicates significance at P ≤ 0.01 and * indicates significance at P≤ 0.05 by Student's t test.

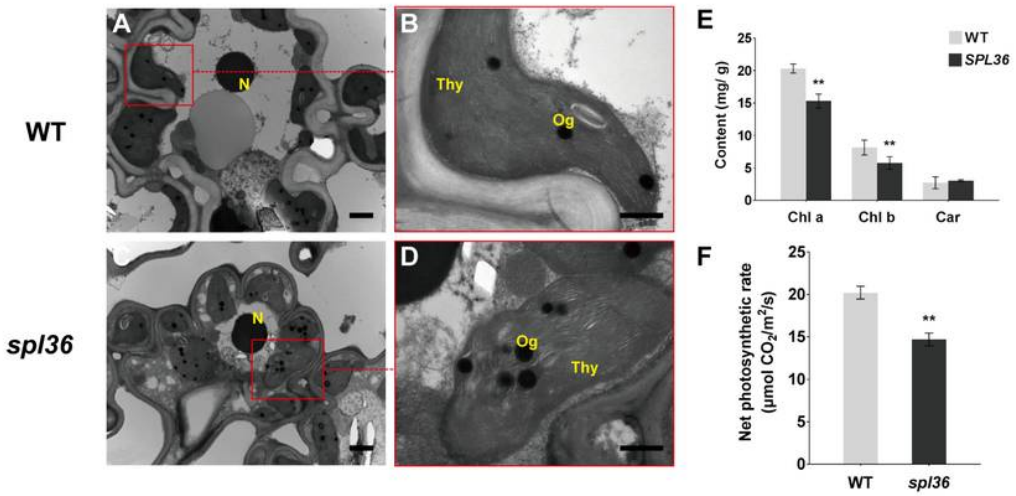


Figure 2

Chloroplast development and Net photosynthetic rate of wild type and mutant A-D Observation of ultrastructure of chloroplasts of wild type and mutant, A,C: leaf cells 6000X; B,D:leaf cells 40000X; N: nucleus; Thy: chloroplast; Og: osmium granules; Bar = 1μm; E Chlorophyll content of leaf of wild type and mutant at tillering stage ; F Wild type and mutant net photosynthetic rate at tillering stage.

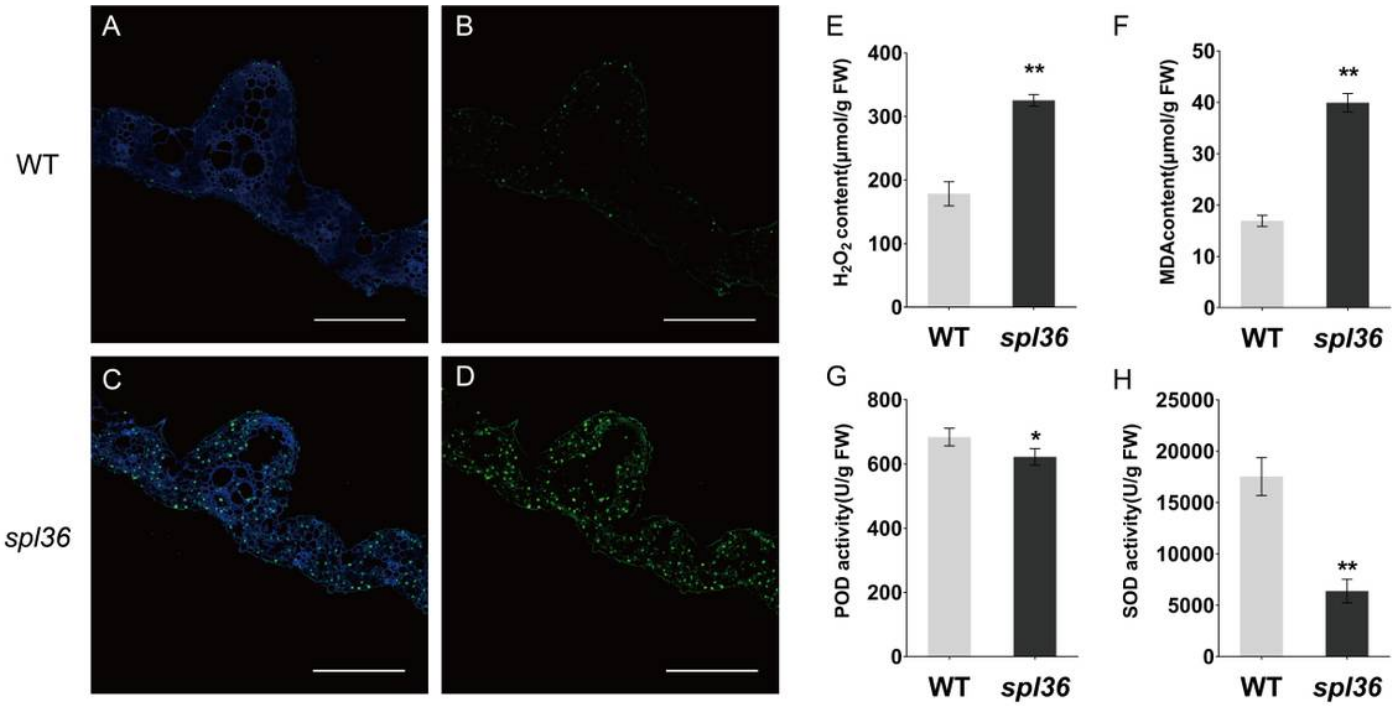


Figure 3

Physiological and biochemical detection of wild-type and mutant A, D TUNEL identification of DNA fragmentation in mesophyll cells. Bar: 100 μm; E, F H2O2, MDA content of mutant spl36 and its wild-type (WT) leaves at heading stage; G, H POD and SOD activities of spl36 and its wild-type (WT) leaves at the heading stage; POD: peroxidase; SOD: superoxide dismutase; MDA: malondialdehyde; WT: wild-type.

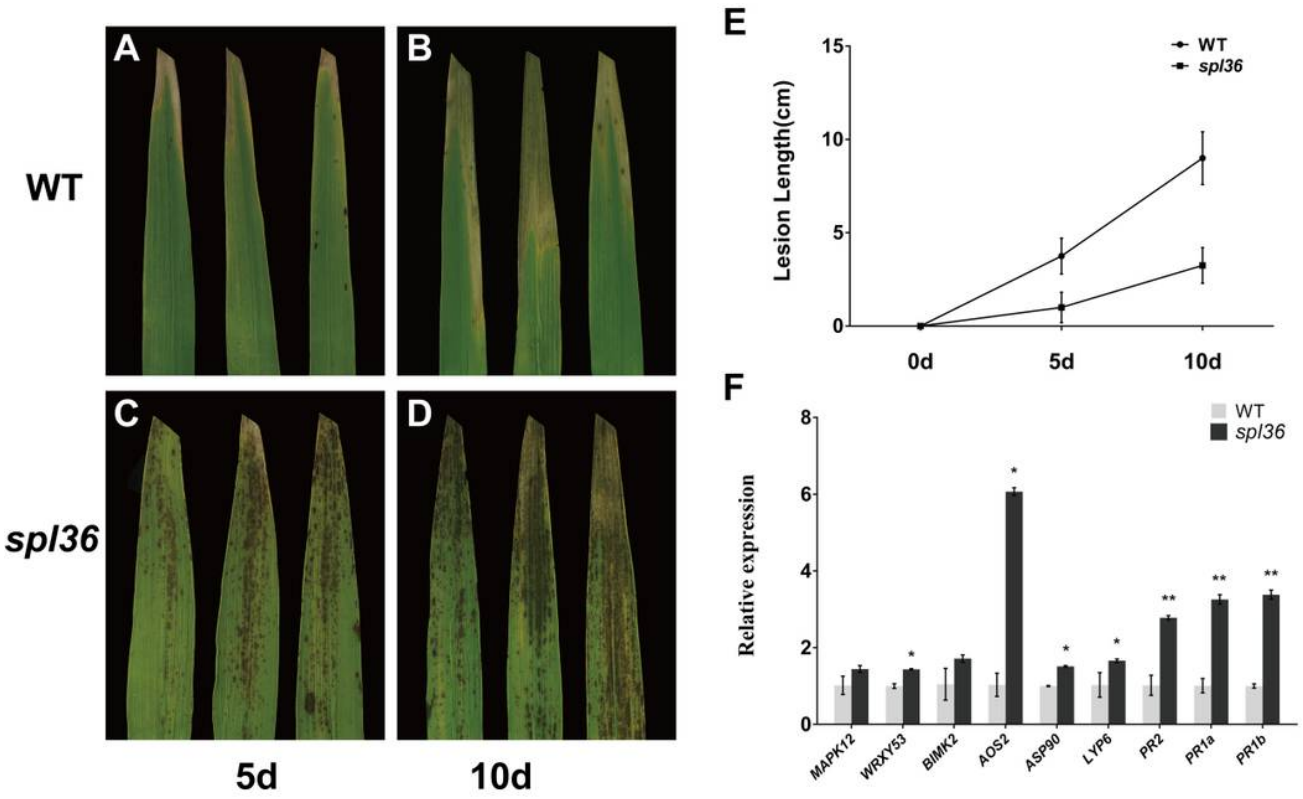


Figure 4

SPL36 Regulates Defense Responses in Rice A-D Phenotypes of wild-type and mutant spl36 after 5 days and 10 days of inoculation; E Statistics of the length of bacterial leaf blight lesions; F Relative expression of defense-related genes.

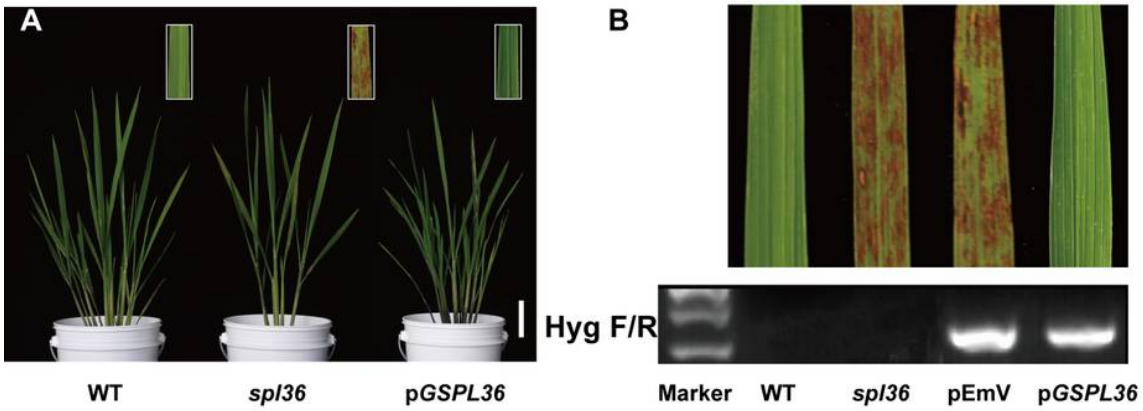


Figure 5

Functional complementation of the spl36 mutant with LOC_Os12g08180 A. spl36 plant transformed with the genomic sequence of SPL36 (pGSPL36) was completely recovered to the wild type phenotype. The insert indicates enlargement of leaf section with lesion spots. B. Transgenic plants were verified by the presence of the hygromycin selectable marker gene. pEmV: the empty vector. Bar: 8 cm in A.

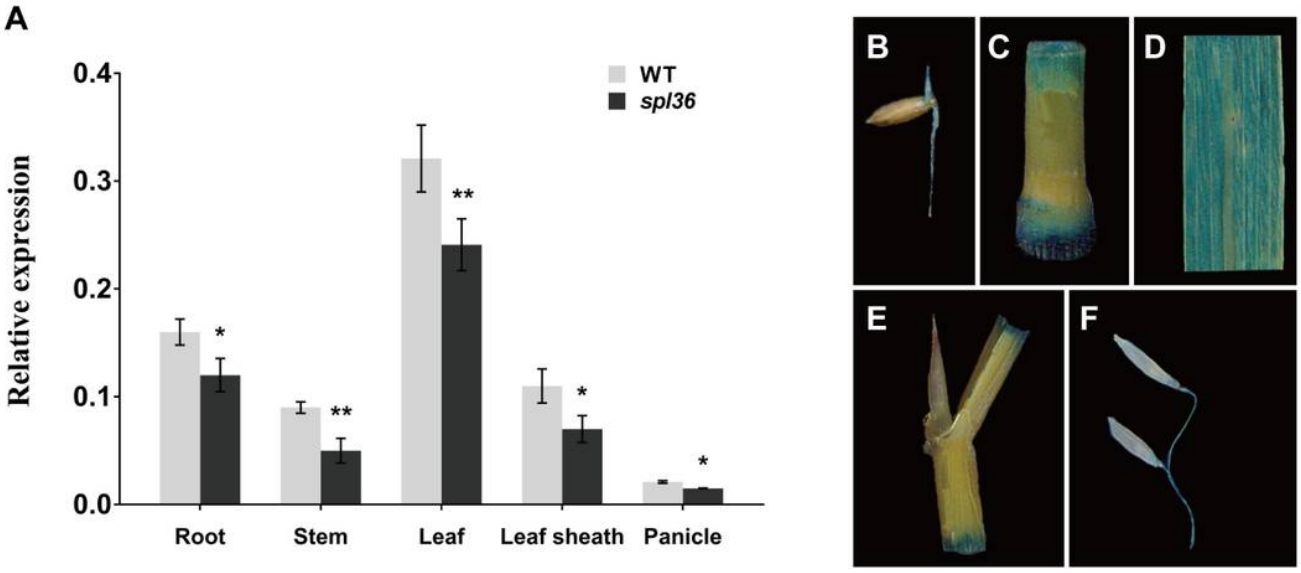


Figure 6

Expression pattern analysis of SPL36 A. Expression of SPL36 in various organs of wild type and mutant spl36 analyzed by quantitative RT-PCR. B-F. Histochemical signals the SPL36 promoter-GUS reporter gene. GUS signals were detected in the root (B), stem (C), leaf (D), leaf sheath (E) and panicle (F).

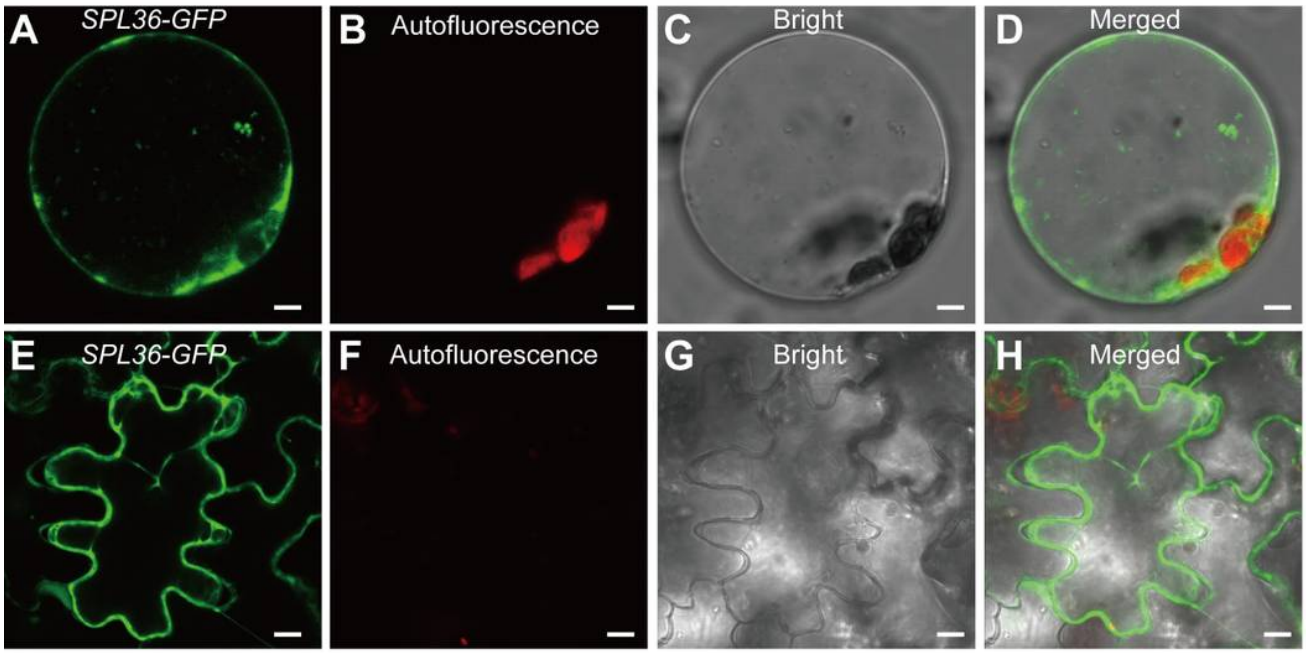
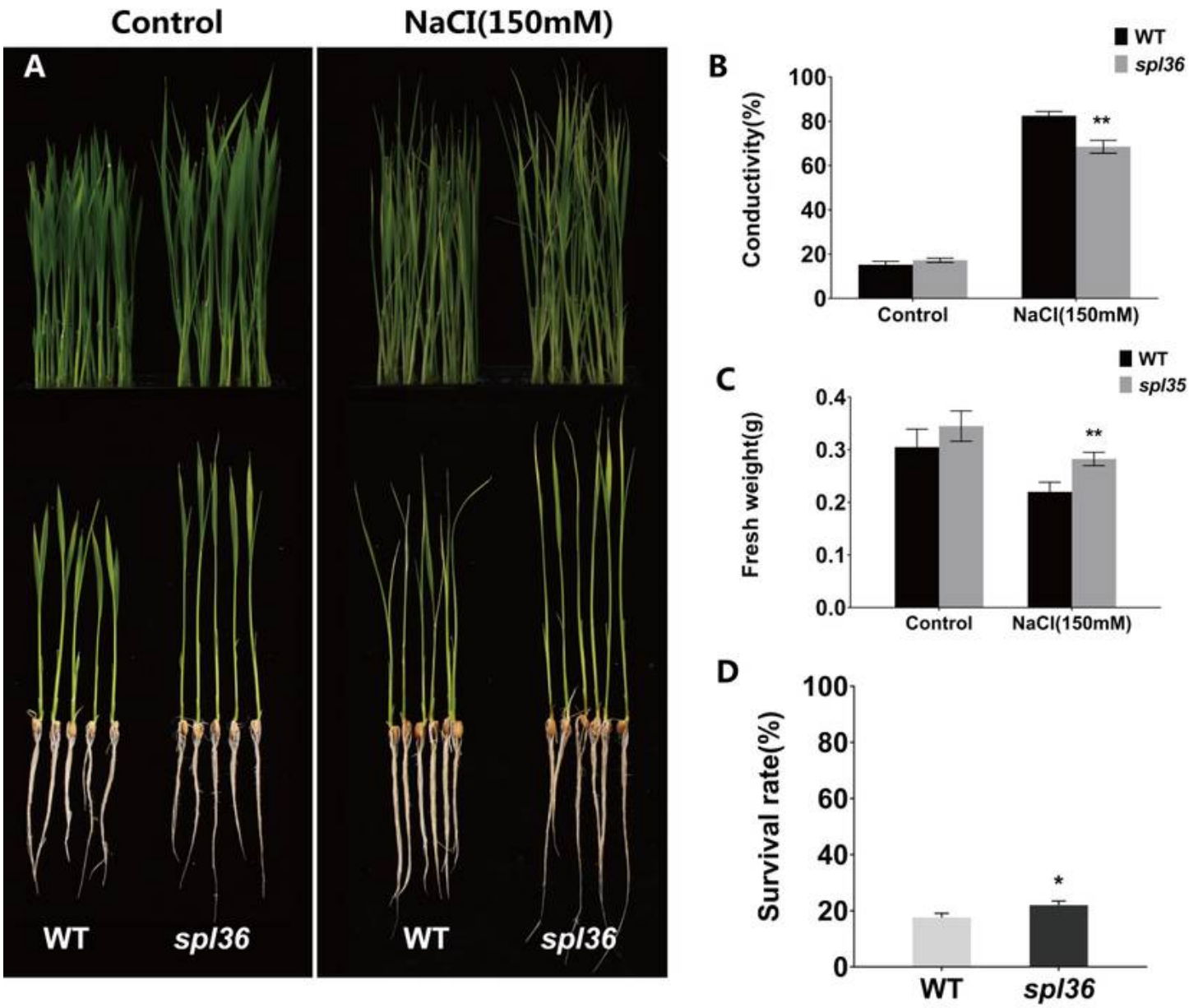


Figure 7

Subcellular localization of SPL36 protein A-D SPL36 is transiently expressed in rice protoplasts; E-H SPL36 is transiently expressed in tobacco, SPL36-GFP: SPL36GFP fusion protein; Bar = 10μm


Control
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A
WT
spl36
WT
spl36
B
WT
spl36
Conductivity(%)
100
80
60
40
20
0
**
Control
NaCl(150mM)
C
WT
spl35
Fresh weight(g)
0.4
0.3
0.2
0.1
0.0
**
Control
NaCl(150mM)
D
Survival rate(%)
100
80
60
40
20
0
*
WT
spl36

Figure 8

Salt stress experiment of wild-type and mutant spl36 at seedling stage A: phenotypes before and after 150mM NaCl treatment of wild-type and mutant seedlings; B: fresh weight before and after 150mM NaCl treatment of wild-type and mutant seedlings; C: conductivity before and after 150mM NaCl treatment of wild-type and mutant seedlings; D: Survival rate of wild-type and mutants after 150mM NaCl treatment

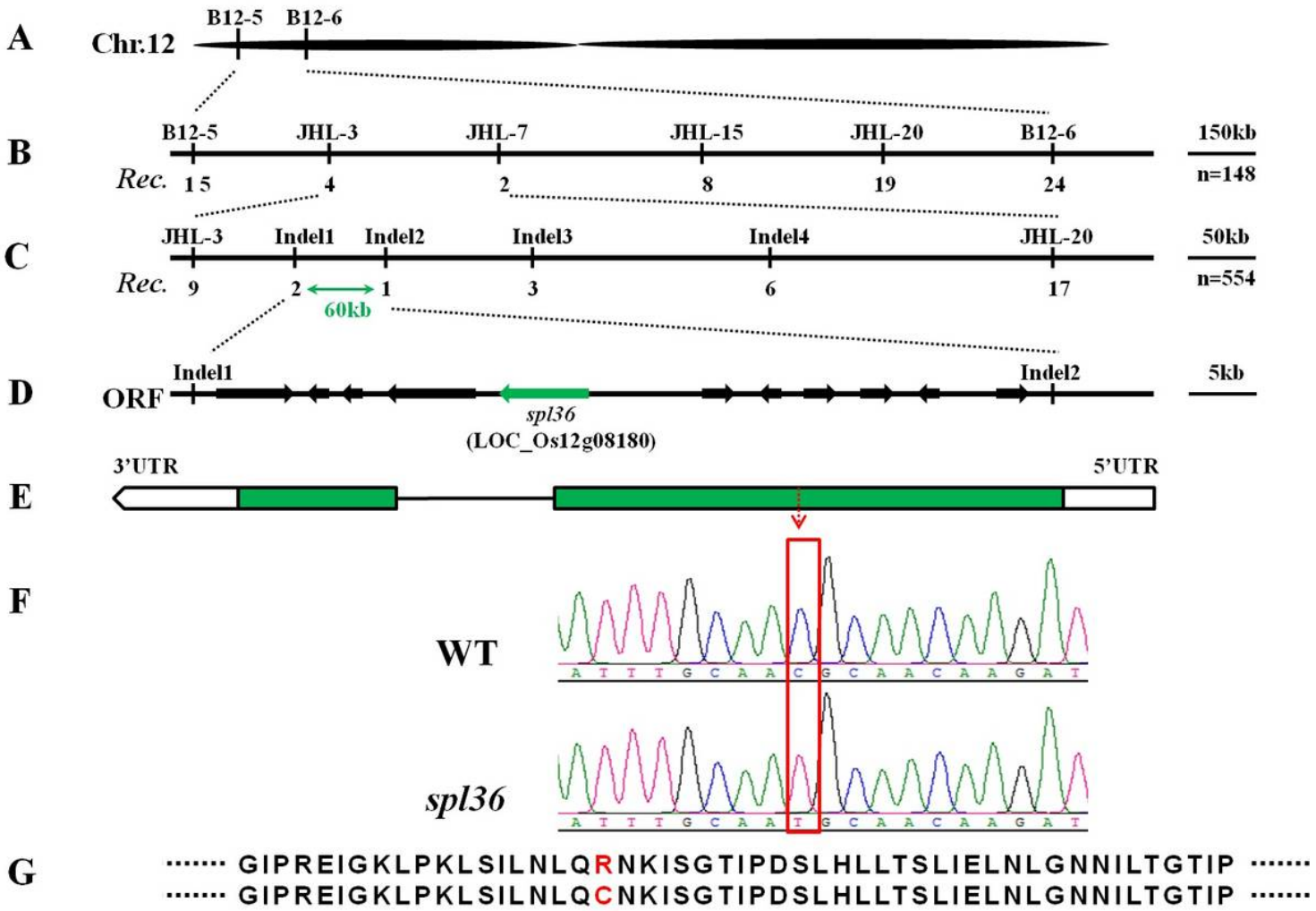

Figure 9

Genetic and physical maps of the SPL36 gene. A The SPL36 gene was located on chromosome 12 between InDel markers B12-5 and B12-6. B The SPL36 gene was delimited to the JHL-3and JHL-7 interval using 148 F2 mutant individuals; marker names and number of recombinants are shown. C Fine genetic mapping of the SPL36 gene based on 554 mutant F2 individuals. D Eleven putative ORFs were located in an ~60-kb region. E Gene structure LOC_Os12g08180. F Sequence analysis of the C-to-T mutation site in plants of wild type and spl36. G Encoded amino acid from Arginine to Cysteine.

Supplementary Files

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