A Blast Resistance Gene *Pi65* with LRR-RLK Domain is Required for Resistance to *M. Oryzae*

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Abstract

Rice blast seriously threatens rice production worldwide. To control this disease, it is necessary to identify and utilize blast resistance genes to breed disease-resistant rice varieties. Here, we report a rice blast resistance gene, *Pi65*, isolated from the resistant variety GangYu129 (abbreviated GY129, *O. sativa japonica*) by map-based cloning. *Pi65* overexpression in the susceptible variety LiaoXing1 (abbreviated LX1, *O. sativa japonica*) enhanced blast resistance, while *Pi65* knockout in GY129 resulted in a decrease in its resistance to rice blast. *Pi65* encodes two transmembrane regions, with 15 LRR domains and one serine/threonine protein kinase catalytic domain, conferring resistance to isolates of *M. oryzae* collected from northeast China. Sixteen amino acids differed between the resistance and susceptibility proteins. The *Pi65* susceptibility allele had one fewer LRR duplication. *Pi65* was constitutively expressed in whole plants, and *M. oryzae* inoculation significantly increased its expression level. Transcriptome sequencing revealed that numerous genes associated with disease resistance were specifically upregulated in GY129 24 h after *M. oryzae* inoculation, and photosynthesis-and carbohydrate metabolism-related genes were particularly downregulated, demonstrating disease resistance gene activation in GY129 mediated by *Pi65* after rice blast fungal infection, cellular basal and energy metabolism was inhibited simultaneously. These combined factors endow GY129 with rice blast resistance. Our study provides genetic resources for improving rice blast resistance in *japonica* rice and enriches the study of rice blast resistance mechanisms.

Key Message

Pi65, a novel gene with a leucine rich-repeat receptor-like kinase (LRR-RLK) domain cloned from *Oryza sativa japonica*, is required for rice blast disease resistance.

Introduction

Rice blast, caused by *M. oryzae*, is a devastating fungal disease in rice worldwide. The annual rice yield loss due to blast damage can be as high as 10 ~ 30% (Skamnioti and Gurr 2009). China is the largest producer of *japonica* rice in the world. The annual planting area of *japonica* rice has reached more than 10 million ha in northern China. The demand for *japonica* rice relative to *indica* rice is increasing each year (Bian et al. 2020). However, rice blast has been a serious threat to *japonica* rice production in northern China for many years. It is widely accepted that breeding and cultivating disease-resistant varieties are the most economic and efficient ways to control rice blast disease. Therefore, it is very important to continue to exploit blast resistance genes. In recent years, scientists have identified several new genes that mediate strong rice blast resistance without affecting rice yield and quality, such as *Pigm*, *Ptr* and *bsrd1* (Li et al. 2017; Zhao et al. 2018; Zhai et al. 2019), and have developed many broad-spectrum resistant varieties; however, most of these genes are derived from *indica* rice, which makes it difficult to use them for the improvement of *japonica* rice resistance in the short term. There has been a lag in rice blast resistance breeding in northern China due to a lack of *japonica* resistance gene resources.

It is important to identify new blast resistance genes from *japonica* rice and to develop resistant germplasm resources in the *japonica* background.

The innate immune system of plants mainly includes two levels of defense. In the first level, when the fungus infects plants, the PAMP (Pathogen-associated molecule pattern) will stimulate the PTI (PAMP triggered immunity) of plants, the pattern recognition receptor on the surface of plant cells specifically recognizes PAMP of pathogenic microorganisms, and pathogenic bacteria or fungi inhibit PTI through effectors; in the second level, the protein encoded by resistance (R) genes in plant cells further recognizes effectors and activates of ETI (Effector triggered immunity) (Liu et al. 2013). The effects of PTI-related resistance genes mediating rice blast resistance are usually moderate and are not species specific (or nonpathogen specific); however, ETI-related resistance genes are usually specific and only recognize certain strains but can mediate a strong resistance response via plant R genes, most of which encode cytoplasmic proteins with nucleotide-binding site-leucine-rich repeat (NLR) domains (Liu et al. 2013).

In 2017, the rice blast resistance gene *Pi65(t)* was finely mapped in the *japonica* rice variety GY129, which is resistant to most *M. oryzae* isolates found in northern China (Zheng et al. 2016). The gene was located on chromosome 11, close to the *Pik* gene cluster and was identified as a new gene differing from other cloned genes. However, its structure and function remain unknown. Here, we show that *Pi65* encodes a leucine rich-repeat receptor-like kinase (LRR-RLK), and we identify 16 SNPs that cause missense mutations between resistance and susceptibility alleles. The resistance function of *Pi65* is further confirmed by showing that the targeted mutation of *Pi65* in GY129 using CRISPR/Cas9 leads to blast susceptibility and that a mutation causing *Pi65* overexpression in LX1 leads to blast resistance. The spatiotemporal expression of *Pi65* and molecular mechanism of disease resistance mediated by the gene were further studied by RT-PCR and RNA sequencing. This study provides genetic resources for the molecular breeding of rice blast resistance in *japonica* rice in particular.

Materials And Methods

Plant materials and disease evaluation

The *japonica* rice variety GY129 is resistant to most *M. oryzae* isolates from Liaoning Province in China, whereas LX1 is susceptible to them (Zheng et al. 2016). In this experiment, the *M. oryzae* isolate QY-13 (ZA1) was used to evaluate disease reactions in the GY129/LX1 BC₁F₂ population.

The germinated rice plants were sown in black plastic containers (10 × 7.0 × 8.5 cm³) with a locally disinfected seedling substrate. The black plastic boxes containing the seedlings were then placed in a blue box one-third full of water (34.5 × 47 × 15 cm³). The seedlings were grown in the greenhouse at 24 to 30°C with an 8 h dark and 16 h light cycle until the V3 to V4 stages, when they were spray-inoculated with spore suspensions (5 × 10⁵ spores/mL) with an airbrush and placed in a blue box with water. Then, the inoculated plants were covered with black plastic sheeting in darkness for 24 h at 25 to 28°C under 100% relative humidity. After culture in the dark, the sunshade net was removed, and the inoculated seedlings

were cultured at 25 ~ 28°C for another 5 days. The punch inoculation of detached rice leaves was performed as follows: 5 µL drops of a spore suspension were placed on three spots on each leaf with a transferpettor, and the leaves were kept in a culture dish containing 0.1% 6-benzylaminopurine (6-BA) in sterile water to provide moisture (Li et al. 2017).

Targeted mutagenesis of Pi65 in GY129 with the CRISPR/Cas9 system

Two potential candidate genes from GY129 were targeted with sgRNA spacers in the anterior segments of their exons. The highly specific sgRNA sequence (Table S1) was designed using CRISPR Design (<http://crispr.mit.edu/>), and PCR was used to anneal sgRNA at 94°C for 5 min and 25°C for 5 min. The CRISPR/Cas9 vector Pcas9 (ZmUbi, OsU6, Hpt) plasmid (Table S2) was linearized by using BsaI (NEB) and connected to the sgRNA with T4 ligase (TIANGEN, NG201). The resulting binary vectors were introduced via electroporation into the *Agrobacterium tumefaciens* strain EHA105. The transformation events were selected based on hygromycin B resistance, and regenerated plants were analyzed for genome editing-induced mutations in the target gene. Chromosomal deletions were detected by PCR with primers located on both sides of each targeted gene.

Pi65 overexpression mutagenesis in LX1

The full-length cDNA of Os11g0694600 was amplified with Primer 1 (Table S1) and cloned into the T vector (pe-Blunt Simple Cloning Vector) to produce T-*Pi65*, which was then recombined with a pCambia1301-UbiN vector to generate the overexpression construct pCambia1301-UbiN-Os*Pi65* (abbreviated as *Pi65*-OE) (Table S2). Regenerated transgenic plants carrying *Pi65*-OE were selected with hygromycin.

Subcellular localization of Pi65 in rice protoplasts

To generate transgenic protoplasts expressing GFP-Pi65, the coding region was amplified using the Pi65 (GFP) primer and cloned into the HBT-sGFP vector under the control of the CaMV 35S promoter (Tables S1 and S2). The protoplasts were extracted from 10-14-day etiolated rice seedlings and then transformed with 10–20 µg of plasmids according to the procedure described in Xiao et al. (2018). The organelle and GFP signals were detected with a Leica microscope (DM4000 B, Germany) using different excitation wavelengths.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from various rice tissues using TRIzol. First-strand cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, RR047A) with an Oligo (dt) 18 primer according to the manufacturer's protocol. qRT-PCR amplification was performed using TB Green™ Premix Ex Taq™ II (Takara, RR820A) and a Roche LightCycler 480 System (CT, USA) following the manufacturer's instructions. qRT-PCR amplification was performed with three biological replications, and the amplified rice Actin1 gene was used as the internal control for gene expression (Table S1). The specific qRT-PCR primers for the *Pi65* gene are listed in Table S2.

Pi65 candidate gene screening and haplotype sequence analysis

We sequenced the candidate genes Os11g0694500, Os11g0694600, Os11g0694850 and Os11g0695000 and analyzed the sequence polymorphisms of the candidate genes to determine the target gene (Table S1). To investigate the distribution of *Pi65* haplotypes in northern *japonica* rice, we tested rice varieties from different areas of northern China (Tables S3 and S4). Primers were used to amplify DNA sequences of candidate genes in different rice varieties using PrimeSTAR® HS (Takara, R040Q); after gel purification, DNA samples were sequenced at Tsingke Biological Technology. The DNA sequences were assembled using DNASTAR. Lasergene.v7.1\SETUP\Editseq software and aligned with DNASTAR. Lasergene.v7.1\SETUP\SeqMan.

Structural and comparative analysis of Pi65

The in silico structural and functional prediction of high-quality assembled sequences was performed at the following sites. Functional annotation was performed after translating the sequence into one of three reading frames. The Simple Modular Architecture Research Tool (<https://smart.embl-heidelberg.de/>) was used for the domain architecture analysis of GY129 and LX1, and tertiary structures were predicted using SWISS-MODEL (<https://swissmodel.expasy.org/>).

Sequence alignments of *Pi65* with other cloned rice blast resistance genes were performed by constructing phylogenetic trees based on sequence similarity. The DNA coding sequences of 24 cloned disease resistance genes, *Pb1*, *Pi1*, *Pi2*, *Pi5-1*, *Pi5-2*, *Pi9*, *pi21*, *Pi35*, *Pi36*, *Pi37*, *Pi54*, *Pi54of*, *Pi54rh*, *Pi63*, *Pib*, *Pia*, *Pid2*, *Pid3*, *Pigm*, *Pikh*, *Pikm-1*, *Pikm-2*, *Pikp-1*, *Pikp-2*, *Piks-1*, *Piks-2*, *Pita* and *Ptr*, were retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). Clustal X was used to carry out multisequence alignments, and the output multisequence alignment results were used to construct phylogenetic trees with MEGA X software.

Transcriptome sequencing of wild-type GY129 and its Pi65 knockout mutants

To study the biological process mediated by *Pi65*, gene expression before and after *Pi65* knockout was compared. Total RNA was extracted from young seedlings of GY129 and the *Pi65* knockout mutant KO-B 6 (abbreviated *Pi65KO*) with three biological replications using TRIzol reagent according to the manufacturer's instructions. cDNA library preparation and sequencing reactions were conducted at the Biomarker Technology Company (Beijing, China). RNA-sequencing (RNA-Seq) analysis was performed following (Zheng et al. 2013). Gene expression levels were measured in the RNA-Seq analyses as the numbers of reads normalized via the reads per kilobase of transcript per million mapped reads (RPKM) method. edgeR software was used to identify differentially expressed genes (DEGs) in pairwise comparisons, and the results of all statistical tests were corrected for multiple tests according to the Benjamini-Hochberg false discovery rate (FDR < 0.05). Sequences were considered to be significantly

differentially expressed if the adjusted P-value obtained was < 0.05 and the fold change (FC, in $\log_2$ FC) in RPKM was > 1.5 between two libraries.

Results

Molecular cloning and functional analysis of Pi65

In our previous work, *Pi65(t)* was mapped between InDel-1 and SNP-4 on the short arm of chromosome 11 (Zheng et al. 2016); within this interval, Os11g0694500, Os11g0694600, Os11g0694850 and Os11g0695000 were found to contain LRR domains. For Os11g0694500 and Os11g0695000, no sequence difference was observed between the parents. Moreover, these two putative genes showed no difference in expression in GY129 and LX1 and could thus be excluded as candidates for *Pi65*. In contrast, the other two candidate genes, Os11g0694600 and Os11g0694850, showed sequence polymorphisms between the two parents. Therefore, Os11g0694600-R and Os11g0694850-R were subjected to further functional analysis.

Using a CRISPR/Cas9 gene mutation strategy, we designed two gene-specific sgRNAs to target Os11g0694600-R and Os11g0694850-R (Figs. 1a and S1a). Agrobacterium-mediated transformation was used to generate 12 and 4 T₀ transgenic lines with the knockout of Os11g0694600-R or Os11g0694850-R, respectively. Six editing types of Os11g0694600-R (KO-B 1–6) (Fig. 1b) and two editing types of Os11g0694850-R (KO-C 1–2) were obtained (Fig. S1). Homozygous mutants for these two genes in generation T₁ were selected for inoculation identification.

Then, we performed rice blast inoculation, and the results showed that in the GY129 background, when Os11g0694850-R was knocked out, the mutants were still resistant to the *M. oryzae* isolate of QY-13 (Fig. 1b). However, when Os11g0694600-R was knocked out, the mutants became susceptible to QY-13. A comparison of the lesion areas of GY129 and *Pi65*KO after inoculation showed that the lesion area of GY129 was significantly smaller than that of *Pi65*KO (Fig. 1b). To further determine the function of Os11g0694600-R, we generated an *Os11g0694600-R* overexpression vector and transformed it into the susceptible rice variety LX1 and transgenic lines were obtained. The rice blast inoculation results indicated that the transgenic lines were all resistant to QY-13 (Fig. 1c). The above results suggested that *Os11g0694600-R* was the rice blast resistance gene *Pi65*.

Sequence structure of Pi65

The full-length cDNA of *Pi65* had a single ORF of 3309 bp, containing 2 introns with lengths of 2923 bp and 386 bp, encoding 1102 amino acids. The deduced protein of Pi65 had 15 LRR domains and one serine/threonine protein kinase domain (Fig. 2a and b). The structural annotation results showed that the *Pi65*R allele (*Os11g0694600-R* in GY129) had one more LRR domain than the *Pi65*S allele (*Os11g0694600-S* in LX1) at sites 543–569. This LRR domain difference was due to the nonsynonymous changes caused by the 16 SNPs between *Os11g0694600-R* and *Os11g0694600-S* (Fig. 2b). To further detect the potential structural differences between *Os11g0694600-R* and *Os11g0694600-S*, we performed

protein structure prediction, and the results revealed that clear structural variation in the 420–580 aa region between the S-allele and R-allele of the *Pi65* gene (Fig. 2c), implying that the R-allele in this region is essential for the rice blast resistance function of the gene.

Intracellular localization of the Pi65 protein

To investigate the intracellular localization of Pi65, we introduced a green fluorescent protein (GFP) construct fused to the coding sequence of Pi65 into rice yellow tissue protoplast cells and examined its intracellular localization using confocal laser scanning microscopy. The GFP-Pi65 fusion protein was also localized to the nucleus and the plasma membrane (Fig. 3a-d and e-h). When GFP was expressed alone, it localized to the nucleus and the plasma membrane (Fig. 3i-l and m-p). In the subcellular localization analysis, the Pi65 proteins colocalized with a nucleus-specific marker and membrane-specific marker.

Comparative analysis of Pi65 and Pi genes

A phylogenetic tree based on sequence similarity was constructed to analyze the structural relationship between *Pi65* and other cloned rice R genes. The comparative analysis of *Pi65* with 24 rice blast resistance genes revealed that *Pi65* appeared to be most closely related to *Pid2*, indicating high homology (orthologous nature) and a close evolutionary relationship (Fig. S1b). *Pi65* was not closely related to other Pi genes, suggesting little or no evolutionary correlation between them.

Distribution of the Pi65 gene in different rice varieties

A previous study indicated that temperate *japonica* is the most rice blast disease-susceptible rice subpopulation. The analysis of *Pi65* alleles and their distribution will help breeders make better use of this rice blast resistance gene. We collected 38 *japonica* rice varieties from 7 regions of China, including Beijing, Xinjiang, Ningxia, Henan, Heilongjiang, Jilin and Liaoning (Fig. S2, Table S3). Through sequence analysis, we identified three haplotypes of *Pi65*, Hap1 (GY129, etc.), Hap2 (LX1, etc.) and Hap3 (JingDao2, abbreviated JD2, etc., *O. sativa japonica*) (Table S4). Hap1 and Hap3 were functional haplotypes (resistant) according to the spray inoculation results. Hap1 was present in 16 varieties that came from Liaoning, Heilongjiang, Henan and Ningxia. Compared with Hap1, Hap3 had a G/A SNP at position 3300, leading to a missense mutation. In addition, 16 SNPs led to missense mutation differences between Hap2 and Hap1, and most rice cultivars carrying Hap2 were susceptible to QY-13, indicating that the SNPs in Hap2 abolished the rice blast resistance function of *Pi65*.

Spatiotemporal expression profile and constitutive expression of Pi65

To investigate *Pi65* expression patterns, we detected the expression of *Pi65* in the GY129 seedling stage (root, leaf and stem) and booting stage (leaf, rachilla, panicle, stem and sheath). The strongest expression was found at the booting stage (16 weeks) in leaves, and there was relatively weak expression at the seedling stage (4 weeks) in roots, young leaves and young sheaths, indicating that *Pi65* is constitutively expressed at different developmental stages and in different organs examined (Fig. 4a). Furthermore, to determine whether the expression of *Pi65* in GY129 could be induced in response to challenge by *M. oryzae*, we inoculated at the three and half-leaf stage-seedlings (4 weeks) of GY129 (resistant variety) and LX1 (susceptible variety) with QY-13 and performed quantitative (q)RT-PCR at six time points (0, 12, 24, 48, 72 and 96 hpi) (Fig. 4b). Relative to the control, *Pi65* expression in GY129 first decreased slightly after inoculation and then increased gradually, reaching a peak at 72 h after inoculation, while in LX1, *Pi65* first decreased and then increased significantly at 48 h after inoculation, after which it decreased again and finally reached a peak at 96 h after inoculation. *Pi65* expression in GY129 was mostly higher than that in LX1 before and after inoculation; however, *Pi65* expression was slightly lower in GY129 than in LX1 only at 48 h after inoculation. It can be concluded that *Pi65* is constitutively expressed and that its expression in disease-resistant varieties is mostly higher than in susceptible varieties both before and after inoculation, with the only difference in this pattern occurring 48 h after inoculation.

Analysis of the rice blast resistance mechanism based on transcriptome sequencing regulated by *Pi65*

To further investigate the regulatory mechanism mediated by *Pi65*, transcriptome sequencing was performed on samples from GY129 and *Pi65*KO mutant plants before and 24 h after inoculation with *M. oryzae*. Genes whose expression levels were increased or decreased by 0.5-fold or more after *M. oryzae* inoculation compared with mock inoculation were identified as DEGs (Fig. 5a). Only those DEGs found in three independent biological replicates were selected for further analyses. A total of 7852 DEGs (2869 upregulated genes and 4983 genes) and 7316 DEGs (2645 upregulated genes and 4671 downregulated genes) were detected in GY129 and the *Pi65*KO mutant, respectively. Among all DEGs, 1617 upregulated DEGs and 3431 downregulated DEGs were shared between WT GY129 and the *Pi65*KO mutant. In addition, 1234 genes and 1525 genes were specifically upregulated and downregulated, respectively, in GY129.

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that the pathways that were most highly enriched in common DEGs in GY129 and *Pi65*KO were associated with metabolic pathways, biosynthesis of secondary metabolites, fatty acid metabolites, phenylpropanoid biosynthesis and ascorbate and aldarate metabolism (Fig. 5b). Gene Ontology (GO) analysis showed that these DEGs were mainly enriched in the categories of “single-organism process” (GO:0044699), “response to stimulus” (GO:0050896), “response to chemical” (GO:0042221), “response to oxygen-containing compound” (GO:1901700) and “biological regulation” (GO:0065007) (Fig. 5c). The results indicated that both GY129 and *Pi65*KO mutant rice presented resistance responses after inoculation with *M. oryzae*.

The analysis of the top 20 GO entries showed that the DEGs that were specifically upregulated in GY129 were mainly involved in the “defense response”, “response to biotic stimulus”, “regulation of response to stress”, “response to other organism”, and “response to external biotic stimulus” (Fig. 6a). Genes related to disease defense accounted for the majority of the DEGs, indicating that many genes related to disease defense were activated in GY129 (with *Pi65*) relative to *Pi65*KO after infection by *M. oryzae*.

The specifically downregulated DEGs in GY129 were mainly involved in “chloroplast”, “photosynthesis” and “single-organism biosynthetic process” pathways, and most of these pathways were associated with amino acid metabolism (three pathways), energy metabolism (one pathway), carbohydrate metabolism (four pathways) and transport and catabolism (two pathways) (Fig. 6b). These results indicated that, relative to *Pi65*KO, GY129 specifically presented decreases in photosynthesis, carbohydrate metabolism and amino acid metabolism after infection by *M. oryzae*, which may be closely related to plant resistance to pathogens.

Discussion

In previous work, *Pi65* was finely mapped to the interval between SNP-2 and SNP-8 located in the region from 30.42 to 30.85 Mb on chromosome 11. In this study, we cloned *Pi65* and verified its function by gene knockout and overexpression. Based on gene structure prediction, we found that *Pi65* was different from most NBS-LRR blast resistance genes in that it contained a typical kinase domain encoding a leucine-rich receptor protein kinase, and its genetic distance from *Pid2* was shown to be relatively short in the rice blast resistance gene cluster analysis. Protein kinases are enzymes with catalytic subunits that transfer the primary (terminal) phosphoric acid of nucleoside triphosphate (usually ATP) to one or more amino acid residues in the protein substrate side chain, resulting in conformational changes that affect protein function (Hanks et al. 1988). The variable amino acids in the motif of LRR determine the specificity of its binding with the interacting protein (Kobe and Eisenhofer 1995). In the tertiary structure, the LRR domain forms an α/β helix, which is located on the surface of the spatial structure of the protein and is involved in the interaction between proteins. This mechanism of action is the basis of the cellular molecular recognition process (Shiu and Bleecker 2001). In this study, we found that *Pi65*-Hap1 (in GY129) has one more LRR domain than *Pi65*-Hap2 (in LX1) from amino acids 543–569, resulting in different tertiary structures of the proteins encoded by the resistance and susceptibility alleles, which may be related to the specific recognition of *M. oryzae*.

To further clarify the molecular mechanism of *Pi65*-mediated blast resistance, we performed transcriptome sequencing to investigate gene expression profiles during the compatible and incompatible interactions of GY129 and the *Pi65*KO mutant with *M. oryzae* isolates. A total of 5093 DEGs were common to the two lines. Among these DEGs, 1617 involved in the biosynthesis of secondary metabolites, fatty acid metabolites and phenylpropanoid biosynthesis were upregulated in both GY129 and the *Pi65*KO mutant after inoculation. The results showed that *M. oryzae* infection influences many of the same physiological processes in GY129 and the *Pi65*KO mutant.

In addition to the shared DEGs, we found significant differences between GY129 and the *Pi65*KO mutant in response to rice blast pathogen infection in genes such as those involved in photosynthesis, carbohydrate metabolism and energy production. Several earlier studies have shown that the allocation of resources toward a defense response occurs at the expense of plant fitness (growth and yield), suggesting that defense-related products are autotoxic or that resistance is energetically costly (Bolton 2009). Reducing the photosynthetic rate to allocate resources to defense against pathogens at the expense of photosynthesis has been suggested to be an effective defense mechanism in early infection stages (Hanssen et al. 2011). Comparative phosphoproteomic analysis revealed that a number of photosynthesis-related phosphoproteins were downregulated in both compatible and incompatible interactions between rice and *M. oryzae* (Li et al. 2015). Similarly, Hanssen et al. (2011) showed that a number of photosynthesis-related genes were downregulated in tomato plants infected with Pepino mosaic virus during early stages of infection. However, in the present study, 100 DEGs associated with plant cell-based metabolism were specifically identified in GY129; these genes were associated with plant cell-based metabolism, including carbohydrate metabolism, lipid metabolism, amino acid metabolism, biosynthesis of other secondary metabolites, energy metabolism, nucleotide metabolism, metabolism of cofactors and vitamins and metabolism of terpenoids and polyketides. In contrast, these DEGs were not found in *Pi65*KO mutant plants, so we suspect that *Pi65* plays an important role in reducing photosynthesis and cellular energy metabolism, which may be important for starving the pathogen and thus limiting its reproduction and expansion.

Additionally, 1234 DEGs that were specifically upregulated in GY129 exhibited enrichment in several GO terms associated with disease defense, such as “defense response” (GO:0006952), “response to stimulus” (GO:0050896), “response to biotic stimulus” (GO:0009607) and “response to stress” (GO:0006950), including 24 disease resistance proteins, 8 E3 ubiquitin-protein ligases, 6 G-type lectin S-receptor-like serine/threonine-protein kinases, 9 L-type lectin-domain containing receptor kinases, 5 mitogen-activated protein kinases, 10 pentatricopeptide repeat-containing proteins, 17 probable LRR receptor-like serine/threonine-protein kinases, 5 probable protein phosphatase 2C proteins, 5 probable serine/threonine-protein kinases, 5 probable WRKY transcription factors, 21 putative disease resistance proteins, 12 receptor kinase-like proteins, 2 serine/threonine-protein phosphatases, and 21 wall-associated receptor kinases. Furthermore, some of these DEGs that were specifically upregulated in GY129 were downregulated in the *Pi65*KO mutant, such as callose synthase (LOC_Os01g34880), Bowman-Birk serine protease inhibitor (LOC_Os01g03330) and stress-response A/B barrel domain-containing protein (LOC_Os07g41810) genes.

In summary, the rice blast resistance gene *Pi65* was identified from *japonica* rice variety GY129, and its disease resistance function was confirmed. *Pi65* encodes a leucine-rich receptor-like protein kinase. The susceptibility allele of *Pi65* has one fewer LRR domain, and the tertiary structure of the encoded protein is significantly different, which may be the key factor whereby *Pi65* confers resistance to rice blast. Transcriptome sequencing results showed that 24 h after rice blast fungus inoculation, a large number of genes associated with disease resistance were upregulated specifically in GY129, and photosynthesis- and carbohydrate metabolism-related genes were simultaneously significantly downregulated, showing

that after rice blast fungus infection, disease resistance genes were activated. At the same time, there was a significant reduction in the basal metabolism of cells, and the combination of these factors endowed GY129 with resistance to rice blast. Our study provides genetic resources for the improvement of rice blast resistance in *japonica* rice and enriches the study of rice blast resistance mechanisms.

Declarations

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Availability of data and material

The data sets generated and analyzed during this study are available on reasonable requests from the corresponding authors.

Code availability

Not applicable.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Li-Li Wang, Zuo-Bin Ma. The first draft of the manuscript was written by Li-Li Wang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

CRISPR/Cas9-mediated mutation of two candidate genes in GY129 and disease reactions of Pi65-overexpressing lines of LX1. **a** The candidate gene Os11g0694600 was knocked out with specific gRNAs. WT, wild-type Os11g0694600 in GY129, and KO-B 1-6, edited types of Os11g0694600. **b** Disease reactions in wild-type GY129 and CRISPR-edited mutant leaves after spray-inoculation with the QY-13 isolate. KO-B and KO-C are Os11g0694600 and Os11g0694850, respectively; lesion lengths were determined on inoculated leaves at 7 days post-inoculation. **c** Blast reactions in GY129, Pi65OE and LX1 plants. Leaves of 4-week-old plants were punch-inoculated; Lesion lengths were determined on inoculated leaves at 7 days post-inoculation.

Figure 3

Intracellular localization of the Pi65 protein. Confocal fluorescence images (b-d, f-h, j-l, n-p) and differential interference contrast (DIC) images (a, e, i, m) of rice yellow tissue protoplasts in cells expressing GFP-Pi65 (a-h) or GFP (i-p). The rice Pi65 protein fused with RFP is a nucleus marker (a-d, i-l) and a membrane marker (e-h, m-p). Scale bar: 5 μ m.

Figure 4

Expression of *Pi65* in different organs and at different time points in both compatible and incompatible interactions. **a** Constitutive expression of *Pi65* in different organs of rice at the seedling stage. **b** Constitutive expression of *Pi65* in different organs of rice at the booting stage. **c** Profiles of *Pi65* expression in GY129 at different time points (0, 12, 24, 48, 72 and 96 hpi) after inoculation detected by qRT-PCR using the relative $-2\Delta\Delta CT$ method with *Actin1* as an internal control. Data represent means with error bars showing $\pm$ s.d. (n=3).

Figure 5

DEG analysis of GY129 and Pi65KO. a Venn diagram analysis of upregulated and downregulated genes in GY129 and Pi65KO cells. b KEGG enrichment analysis of all DEGs in GY129 and Pi65KO, the X-axis represents the RichFactor, and the left side of the Y-axis represents KEGG pathways. c GO enrichment analysis for all DEGs in GY129 and Pi65KO, the X-axis represents the $-\log_{10}(\text{Pvalue})$, and the left side of the Y-axis represents GO item types.

Figure 6

GO enrichment analysis of GY129 of DEGs. a GO enrichment analysis of up-DEGs in GY129, X-axis represents the $-\log_{10}(\text{Pvalue})$, the left side of the Y-axis represents GO term types. b GO enrichment analysis of down-DEGs with GY129, the X-axis represents the $-\log_{10}(\text{Pvalue})$, the left side of the Y-axis represents GO term types.

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