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ORIGINAL ARTICLE

Cloning and functional analysis of the novel rice blast resistance gene *Pi65* in *japonica* rice

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Abstract

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

Abstract Rice blast seriously threatens rice production worldwide. Utilizing the rice blast resistance gene to breed rice blast-resistant varieties is one of the best ways to control rice blast disease. Using a map-based cloning strategy, we cloned a novel rice blast resistance gene, *Pi65*, from the resistant variety GangYu129 (abbreviated GY129, *Oryza sativa japonica*). Overexpression of *Pi65* in the susceptible variety LiaoXing1 (abbreviated LX1, *Oryza sativa japonica*) enhanced rice blast resistance, while knockout of *Pi65* in GY129 resulted in susceptibility to rice blast disease. *Pi65* encodes two transmembrane domains, with 15 LRR domains and one serine/threonine protein kinase catalytic domain, conferring resistance to isolates of *Magnaporthe oryzae* (abbreviated *M. oryzae*) collected from Northeast China. There were sixteen amino acid differences between the *Pi65* resistance and susceptible alleles. Compared with the *Pi65*-resistant allele, the susceptible allele exhibited one LRR domain deletion. *Pi65* was constitutively expressed in whole plants, and it could be induced in the early stage of *M. oryzae* infection. Transcriptome analysis revealed that numerous genes associated with disease resistance were specifically upregulated in GY129 24 h post inoculation (HPI); in contrast, photosynthesis and carbohydrate metabolism-related genes were particularly downregulated at 24 HPI, demonstrating that disease resistance-associated genes were activated in GY129 (carrying *Pi65*) after rice blast fungal infection and that cellular basal metabolism and energy metabolism were inhibited simultaneously. Our study provides genetic resources for improving rice blast resistance and enriches the study of rice blast resistance mechanisms.

Introduction

Rice blast, caused by *M. oryzae*, is a devastating fungal disease 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 hectares (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 way 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,

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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. Although the substitution of genetic background can be achieved through multiple backcrosses, this is time consuming and difficult to apply in resistant breeding. Rice blast resistance breeding in northern China has lagged behind due to a lack of 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 systems of plants mainly include two levels of defense. In the first level, when the fungus infects plants, the PAMP (pathogen-associated molecule pattern) stimulates the PTI (PAMP triggered immunity) of plants, the pattern recognition receptor on the surface of plant cells specifically recognizes the 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 ETI (effector triggered immunity) (Ronald and Beutler 2010; Cheng et al. 2012; Liu et al. 2013). PTI is a nonspecific defense response that is characterized by a broad resistance spectrum and persistent resistance. The pathogen-related molecular patterns that stimulate PTI, such as polysaccharides, polypeptides and flagellin, are widely conserved in pathogens (or are considered not to be pathogen-specific) (Ronald and Beutler 2010; Chen and Ronald 2011; Segonzac and Zipfel 2011); however, ETI-related resistance genes are usually specific and recognize limited strains, but can mediate a strong resistance response, and most of them 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 from 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 distinct 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* was further confirmed by both CRISPR/Cas9-mediated gene knockout mutation in the resistant rice variety GY129 and overexpression of *Pi65* in the susceptible rice variety LX1. The spatiotemporal expression of *Pi65* and the 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 and enriches the study of rice blast resistance mechanisms.

Materials and methods

Plant materials and disease evaluation

The *japonica* rice variety GY129 is resistant to most of the tested *M. oryzae* isolates (e.g., ZA1, ZA9, ZB1, ZB13, ZC1, ZE1, ZF1 and ZG1) 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 selected to evaluate the disease reactions of the GY129/LX1 BC_1F_2 population and *Pi65* knockout and overexpressed mutants. The donor variety GY129 is used in international research and breeding (Mukhina et al. 2020).

The rice plants were sown in black plastic containers ($10 \times 7.0 \times 8.5$ cm^3) 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 ($34.5 \times 47 \times 15$ cm^3). The seedlings were grown in a greenhouse at 24 to 30 °C with an 8 h dark and 16 h light cycle until the three and half leaves stages (approximately three weeks old). We spray-inoculated spore suspensions (5×10^5 spores/mL) and placed them in a blue box. 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 was removed, and the cells 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/). 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 at *BamHI* to generate the overexpression construct pCambia1301-UbiN-Os*Pi65* (abbreviated as OE-*Pi65*) (Table S2). The construct was transformed into calli of *Oryza sativa japonica* LX1 mediated by *Agrobacterium tumefaciens*. The transgenic plants were screened with a solution containing 300 mg/mL carbenicillin and 50 mg/mL hygromycin, and the hygromycin resistance gene was detected by PCR. All transgenic plants were properly managed in an artificial climate incubator in Liaoning Province. More than 20 transgenic lines were obtained, and 3 independent T_2 lines were used in this study.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from 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 rice Actin1 gene was used as the internal control for gene expression analysis (Table S1). The *Pi65* gene-specific qRT-PCR primers are listed in Table S1.

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 *japonica* rice, we tested *japonica* rice varieties from different areas of northern China (Table 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/).

The peptide sequences encoded by *Pi65* were subjected to search strings in the nonredundant protein Rice Information GateWay (RIGW) database (https://rice.hzau.edu.cn/rice_rs3/), and the top 24 high-similarity homologous genes were downloaded for further analyses. Hmmer software was used to identify the LRR and kinase domain of each LRR-RLK gene. Clustal X was used for multisequence alignments, and the phylogenetic tree was constructed using a neighbor-joining method in MEGA software (Saitou and Nei 1987; Kumar et al. 2018).

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

To study the biological processes mediated by *Pi65*, we analyzed the *Pi65*-associated gene expression pattern. Total RNA was extracted from young seedlings of GY129 (carrying the *Pi65* gene) and the *Pi65* knockout mutant KO-B 6 (abbreviated KO*Pi65*) with three biological replicates 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). Genes were considered to be significantly differentially expressed if the adjusted *P*-value was < 0.05 and $\log_2FC \geq 2$ and $\log_2FC \leq -2$ between two libraries.

Results

Molecular cloning and functional analysis of *Pi65*

In our previous work, *Pi65(t)* was localized in a 430 kb region between InDel-1 and SNP-4 on the short arm of chromosome 11 (Zheng et al. 2016). Within the interval,

we found that four genes, Os11g0694500, Os11g0694600, Os11g0694850 and Os11g0695000, all contain typical LRR domains. Sequence analysis results showed that for Os11g0694500 and Os11g0695000, there were no sequence differences between the resistant parent GY129 and the susceptible parent LX1. However, in the other two candidate genes, Os11g0694600 and Os11g0694850, sequence polymorphisms were present between the resistant and susceptible parents. Therefore, *Os11g0694600-R* and *Os11g0694850-R* were used for further functional analysis.

Using a CRISPR/Cas9 gene mutation strategy, we designed two gene-specific sgRNAs to target Os11g0694600-R and Os11g0694850-R (Fig. 1a and S1a). We obtained a total of 12 and 4 independent T_0 CRISPR knockout transgenic lines for Os11g0694600-R and 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 T_2 were selected for further rice blast resistance evaluation. The rice blast inoculation 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 KO*Pi65* after inoculation showed that the lesion areas of KO*Pi65* were significantly larger

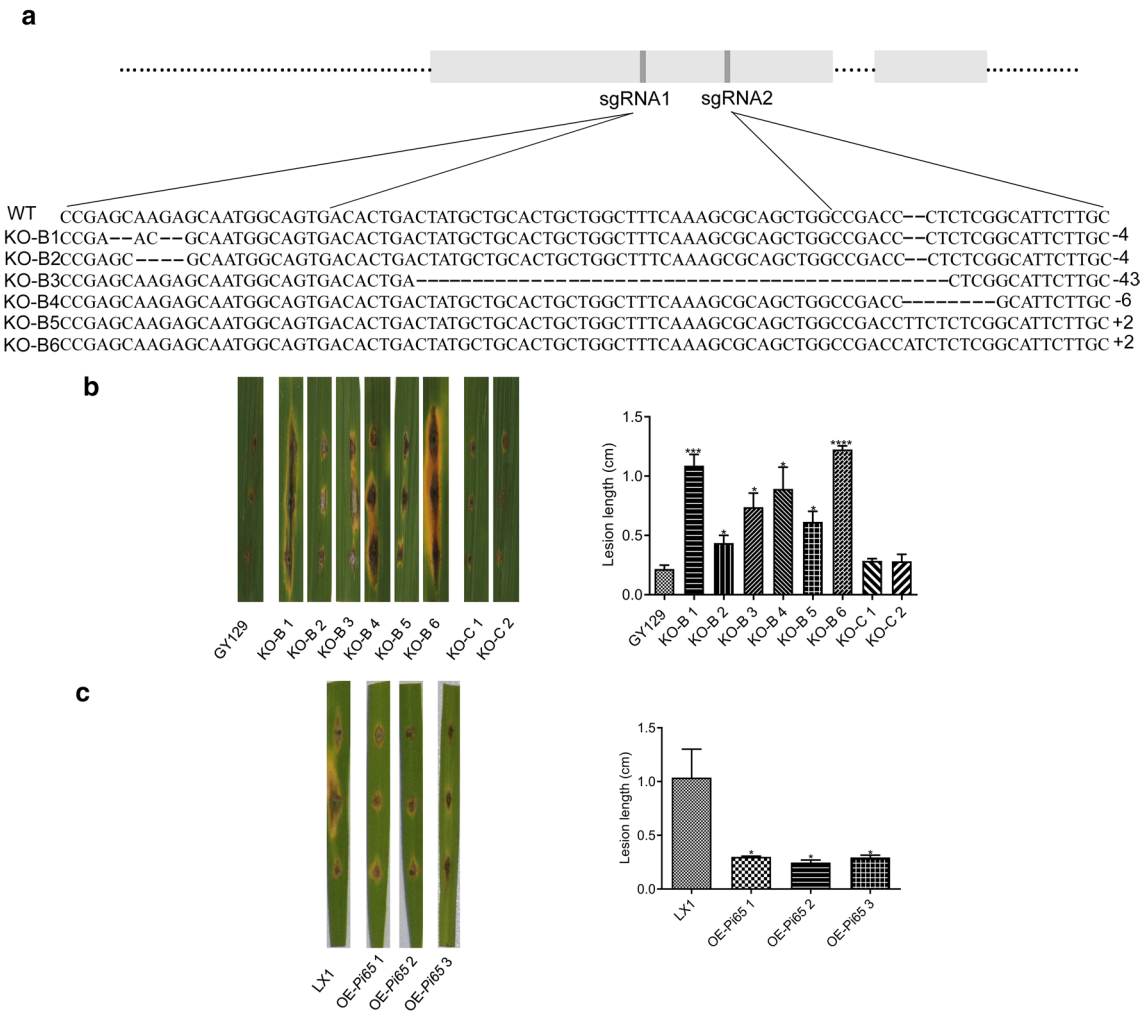

Fig. 1 CRISPR/Cas9-mediated mutation of two candidate genes in GY129 and disease reactions of *Pi65*-overexpressing lines of LX1. **a** The candidate gene Os11g0694600 was knocked out with specific gRNAs. WT, wild-type Os11g0694600 in GY129, and KO-B 1-6, edited types of Os11g0694600. **b** Disease reactions 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 (Student's *t*-test; $*P<0.05$, $***P<0.001$, $****P<0.0001$). **c** Blast reactions in OE-*Pi65* and LX1 plants. Leaves of 4-week-old plants were punch-inoculated; lesion lengths were determined on inoculated leaves at 7 days post-inoculation (Student's *t*-test; $*P<0.05$)

than those of GY129 (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. The rice blast inoculation results indicated that overexpression of *Pi65* in LX1 could enhance its resistance to the *M. oryzae* isolate QY-13 (Fig. 1c). Although R gene overexpression often leads to nonspecific resistance, a combination of wild-type, knockout mutant, and overexpressed lines suggests that these phenotypic changes are indeed due to *Pi65*, demonstrated that *Os11g0694600-R* is the bona-fide rice blast resistance gene of *Pi65*.

Sequence structure of *Pi65*

Pi65 contains 2 introns with lengths of 2,923 bp and 386 bp. The full-length cDNA of *Pi65* has a 3,309 bp open reading frame (ORF), encoding 1,102 amino acids (aa). The Pi65 protein has 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 aa sites 543 to 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,

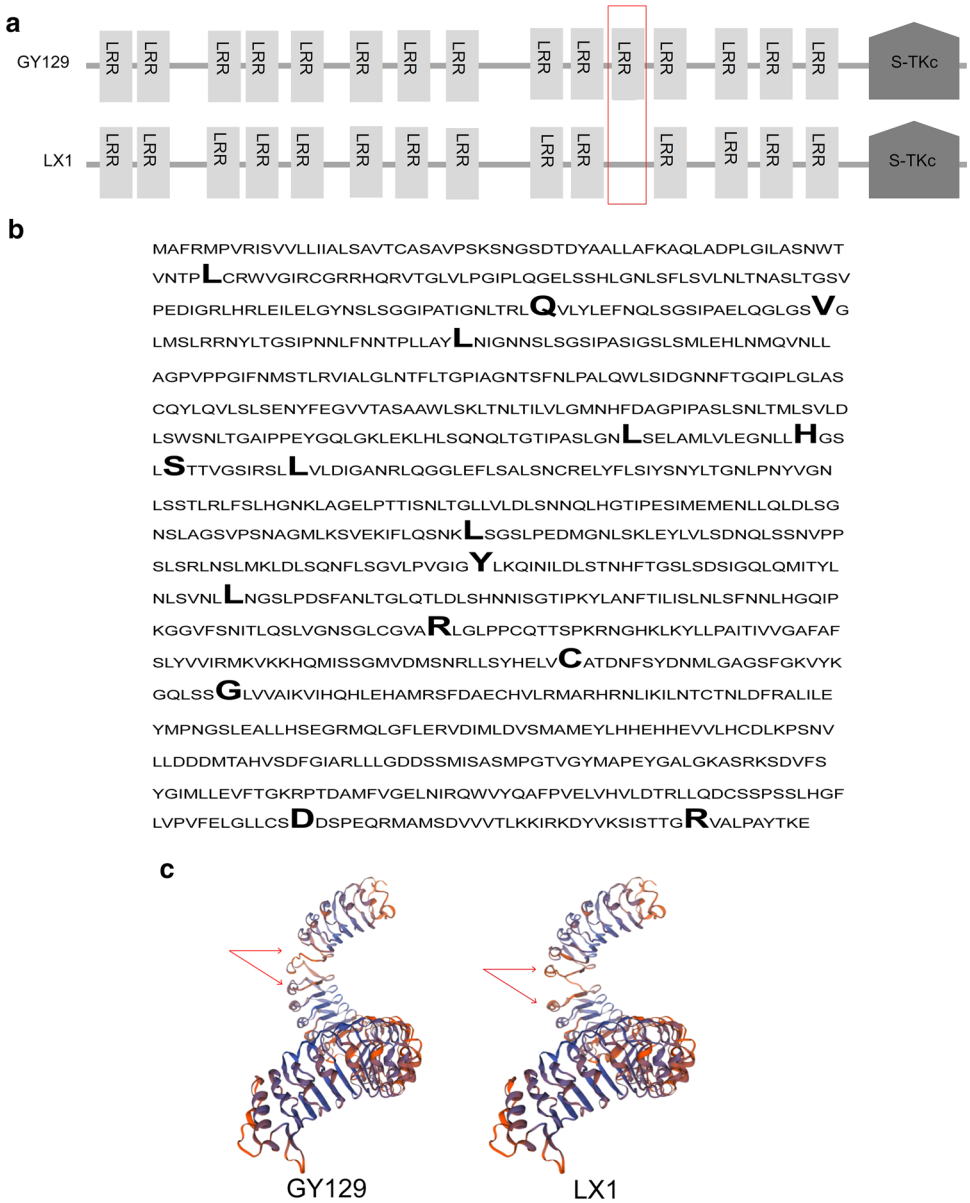

Fig. 2 Structure of *Pi65* and its deduced amino acid sequence. **a** LRRs and serine/threonine protein kinases are shown in the CDS. **b** Deduced peptide sequence encoded by *Pi65*. The bold and enlarged amino acid sequences are specifically present in GY129 and not in LX1. **c** Red arrows indicate the tertiary structural differences in *Pi65* between GY129 and LX1 (colour figure online)

and the results revealed significant structural variation in the 420-580 aa regions 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.

Pi65 phylogeny

The top 24 genes with high homology with *Pi65* were found in the RIGW gene bank for the construction of the evolutionary tree (Fig. S1b). These 25 genes were divided into four groups according to their kinships. Group I contained *Pi65* and another two genes, LOC_Os11g47210 and LOC_Os11g46980, and LOC_Os11g47210 is the previously cloned *Bacterial Blight* resistance gene *Xa26* (Yang et al. 2003). LOC_Os11g46980 is involved in the response to high temperature, and its LRR domain is important for the perception of elevated temperature (Zhang et al. 2011). It is interesting that the closest gene to *Pi65* is *Xa26*. Further study of these two genes will help us to better understand their resistance differentiation.

Distribution of *Pi65* and its alleles in different rice varieties

A previous study indicated that temperate *japonica* is the most rice blast disease-susceptible rice subpopulation (Kang et al. 2016). 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., *Oryzasativa japonica*) (Table S4). Hap1 and Hap3 were functional haplotypes (resistant) according to the spray inoculation results. Hap1 was present in 15 varieties that came from Liaoning, Heilongjiang, Henan and Ningxia. Compared with Hap1, Hap3 had a G/A SNP at position 3264, 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 of *Pi65*

To investigate *Pi65* expression patterns, we detected the expression of *Pi65* in the GY129 seedling (root, leaf and stem) and booting (leaf, rachilla, panicle, stem and sheath) stages. The strongest expression was found in the booting stage (16 weeks) in leaves, and there was relatively weak expression in 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 tested organs (Fig. 3a). Furthermore, to determine whether the expression of *Pi65* in GY129 could be induced during *M. oryzae* infection, we performed the rice blast inoculation and conducted quantitative (q) RT-PCR analysis at six time points (0, 12, 24, 48, 72 and 96 HPI) (Fig. 3b) using the three- and half-leaf stage seedlings of GY129 (resistant variety) and LX1 (susceptible variety) as the materials. During the early stage (12 HPI) after *M. oryzae* inoculation, the expression level of *Pi65* was significantly increased in GY129 compared to the control (mock treatment), reaching a peak at 72 HPI. However, in LX1, *pi65* (*Pi65*'s susceptible

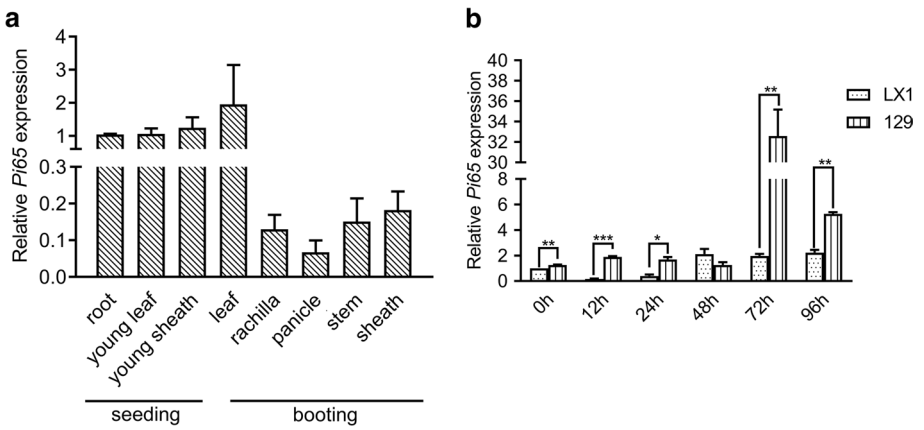

Fig. 3 Expression of *Pi65* in different organs and at different time points in both compatible and incompatible interactions. **a** Constitutive expression of *Pi65* in different organs of rice at the seedling stage. Constitutive expression of *Pi65* in different organs of rice at the booting stage. **b** 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 ± s.d. ($n = 3$) (Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

allele) decreased in early stages (both 12 and 24 HPI) and then increased at 48 HPI. In brief, the expression level of *Pi65* in GY129 was higher than that in LX1 before and after inoculation, except at 48 HPI. In conclusion, *Pi65* is constitutively expressed, and its expression could be quickly induced in resistant rice varieties but could not be induced in the early stage in susceptible rice varieties.

Transcriptome analysis of the expression pattern of *Pi65*-associated genes

To further investigate the regulatory mechanism mediated by *Pi65*, transcriptome sequencing was performed on samples from GY129 and KO*Pi65* mutant plants. Only those DEGs found in three independent biological replicates were selected for further analyses. A total of 2,709 DEGs (619 upregulated genes and 2,090 downregulated genes) and 2,221 DEGs (299 upregulated genes and 1,922 downregulated genes) were detected in GY129 and the KO*Pi65* mutant, respectively (Fig. 4a). Among all DEGs, 128 upregulated DEGs and 1,399 downregulated DEGs were shared between GY129 and the KO*Pi65* mutant. In addition, 489 genes and 690 genes were specifically upregulated and downregulated, respectively, in GY129.

Gene Ontology (GO) analysis showed that these DEGs were mainly enriched in the categories of "single-organism process" (GO:0,044,699), "response to stimulus" (GO:0,050,896), "response to chemical" (GO:0,042,221), "response to oxygen-containing compound" (GO:1,901,700) and "biological regulation" (GO:0,065,007) (Fig. 4b). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that the pathways that were most highly enriched among all DEGs in GY129 and KO*Pi65* were associated with metabolic pathways, biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, cutin, suberin and wax biosynthesis and fatty acid metabolites (Fig. 4c). The results indicated that although both GY129 and KO*Pi65* mutants presented resistance responses after inoculation with *M. oryzae,* the resistance responses in GY129 were much stronger than those in KO*Pi65*.

Additionally, 489 DEGs that were specifically upregulated in GY129 exhibited enrichment in several GO terms associated with disease defense, such as "response to oxygen-containing compound" (GO:1,901,700),"response to chemical"(GO:0,042,221),"response to stimulus"

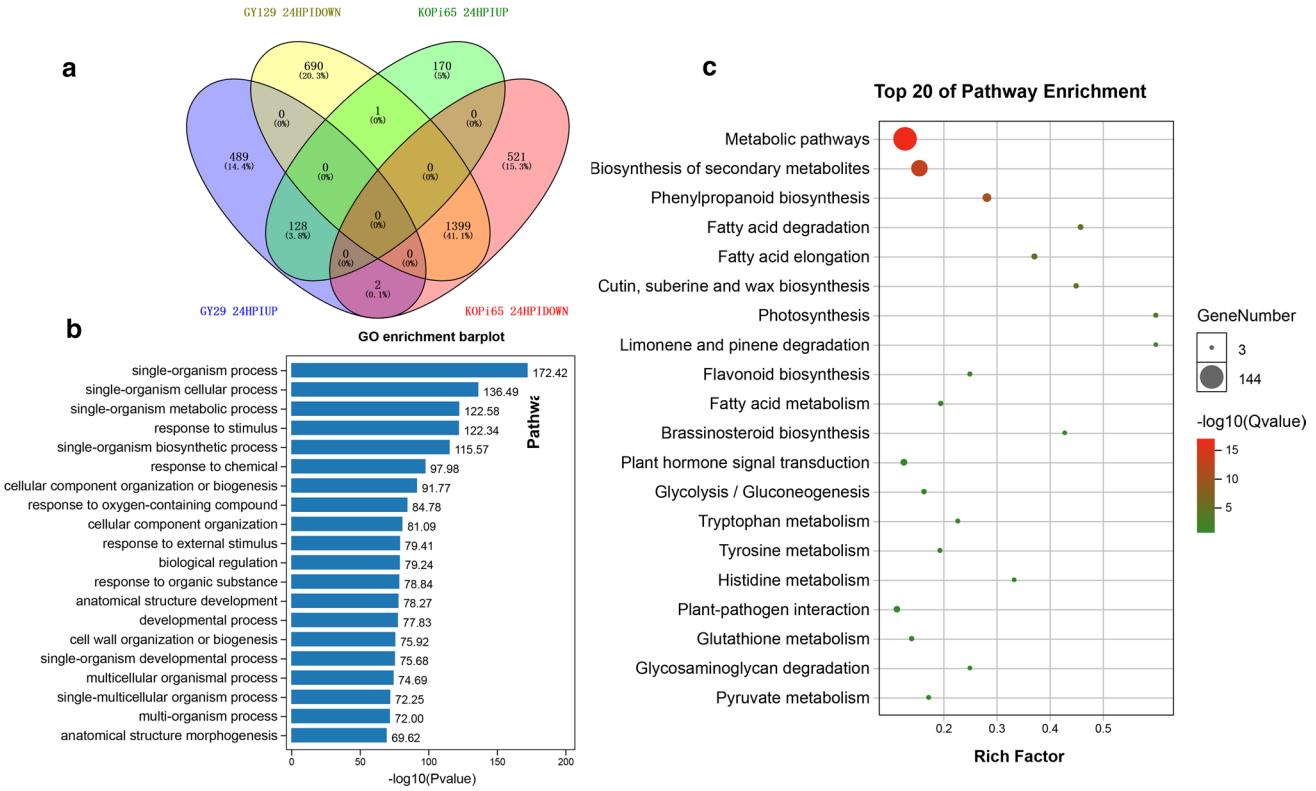

Fig. 4 DEG analysis of GY129 and KO*Pi65*. **a** Venn diagram analysis of upregulated and downregulated genes in GY129 and KO*Pi65* at 24 HPI. **b** GO enrichment analysis for all DEGs in GY129 and KO*Pi65*. The *X*-axis represents the -log10 (*P*value), and the left side of the *Y*-axis represents GO item types. **c** KEGG enrichment analysis of all DEGs in GY129 and KO*Pi65*. The *X*-axis represents the Rich Factor, and the left side of the *Y*-axis represents KEGG pathways

(GO:0,050,896),"defense response" (GO:0,006,952), "response to stress" (GO:0,006,950) and "response to fungus" (GO:0,009,620), including 10 disease resistance proteins (LOC_Os11g12340, etc.), 6 E3 ubiquitin-protein ligases, 6 G-type lectin S-receptor-like serine/threonine-protein kinases, 15 transcription factors (LOC_Os04g43680, LOC_Os11g02520, LOC_Os11g45740, etc.), 6 L-type lectin-domain containing receptor kinases, 2 pentatricopeptide repeat-containing proteins, 13 probable LRR receptor-like serine/threonine-protein kinases, 5 probable protein phosphatase 2C proteins, 1 probable serine/threonine-protein kinase, 4 putative disease resistance proteins, 1 receptor kinase-like protein, 1 serine/threonine-protein phosphatase, and 23 wall-associated receptor kinases (LOC_Os02g42150, LOC_Os09g38850, etc.). Furthermore, some of these DEGs that were specifically upregulated in GY129 were downregulated in the KO*Pi65* mutant, such as Bowman-Birk serine protease inhibitor (LOC_Os01g03330) and PPR repeat family (LOC_Os07g41810) genes (Fig. 5a and Tables S5). OsRSR1 (LOC_Os11g12340) and OsRLCK5 can enhance the resistance of rice to sheath blight by regulating ROS balance through the ascorbate-glutathione circulation system (Wang et al. 2021). LOC_Os04g43680, LOC_Os11g02520 and LOC_Os11g45740 are MYB and WRKY transcription factors, respectively, which are involved in rice blast resistance (Wang et al. 2007; Cao et al. 2015; Kishi-Kaboshi et al. 2018). LOC_Os02g42150 and LOC_Os09g38850 are wall-associated receptor kinase genes, both of which are positive regulatory factors of rice resistance to *M. oryzae* infection (Delteil et al. 2016).

The specifically downregulated DEGs in GY129 were mainly involved in the "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. 5b). These results indicated that, relative to KO*Pi65*, 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 *M. oryzae* (Table S6).

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 confirmed that, unlike most NBS-LRR blast resistance genes, it contained a typical kinase domain encoding a leucine-rich receptor protein kinase. Phylogeny studies showed that *Pi65* was closest to *Xa26*. Similarly, the differences between *Xa26* and its alleles are mainly within the xxLxLxx as well (Li et al. 2012). 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; meanwhile, the expression level of *Pi65* in GY129 was higher than that of its allele in LX1. Therefore, the specific structure of *Pi65* and the higher level of gene expression may represent the key mechanism that enforces *Pi65* resistance to rice blast.

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 KO*Pi65* mutant with *M. oryzae* isolates. A total of 1,530 DEGs were common to the two lines. Among these DEGs, 128 involved in the biosynthesis of secondary metabolites, fatty acid metabolites and phenylpropanoid biosynthesis were upregulated in both GY129 and the KO*Pi65* mutant after inoculation. The results showed that *M. oryzae* infection influences many of the same physiological processes in GY129 and the KO*Pi65* mutant.

In addition to the shared DEGs, we found significant differences between GY129 and the KO*Pi65* 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

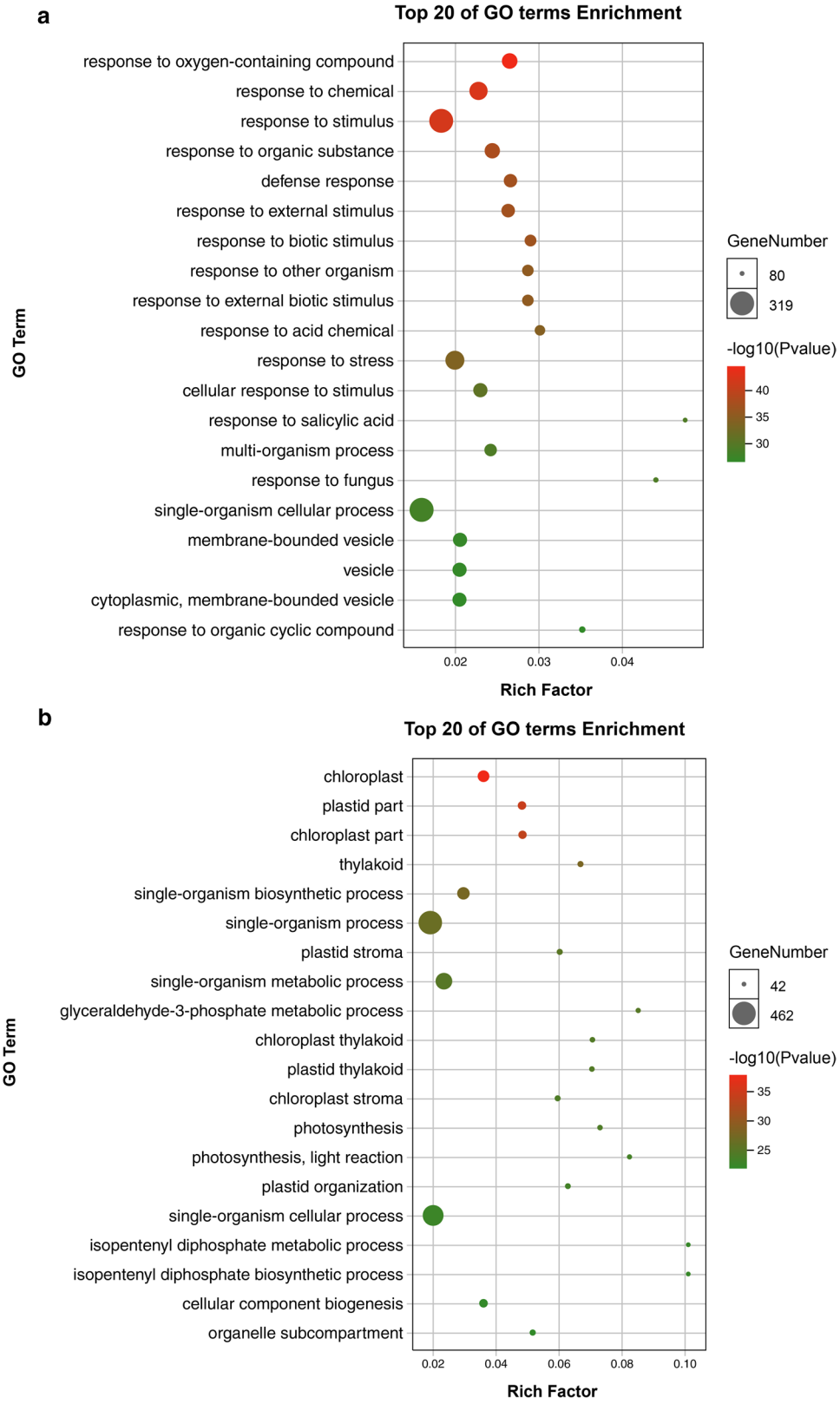

Fig. 5 GO enrichment analysis of GY129 of DEGs. **a** GO enrichment analysis of specifically up-DEGs in GY129, where the *X*-axis represents the Rich Factor, and the left side of the *Y*-axis represents GO term types. **b** GO enrichment analysis of specifically down-regulated DEGs with GY129. The *X*-axis represents the Rich Factor, and the left side of the *Y*-axis represents GO term types

early stages of infection. However, in the present study, 112 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 KO*Pi65* 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.

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", "response to external biotic stimulus", "response to salicylic acid" and "response to fungus" categories. 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*) 24 HPI.

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 disease resistance genes were activated after rice blast fungus infection. 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.

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Author contribution statement LW, WZ, DM, and ZM conceptualized and designed the experiments; MZ, SG, and CW prepared the materials. LW conducted the experiment; LW, ZM, MZ, SG, ZM and CW acquired data; LW, ZM, HW, YB and GS analyzed and interpreted the data; LW drafted the manuscript; and LW, ZM, MZ, SG, ZM, CW, HW, YB, GS, DM and WZ read, revised and approved the manuscript. All authors edited, reviewed, and approved the final version of the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Data availability The data sets generated and analyzed in this study are available upon reasonable request from the corresponding authors.

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