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Cloning and Function Analysis of a Novel Rice Blast Resistance Gene Pi65 in Japonica Rice

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Title

Cloning and function analysis of a novel rice blast resistance gene *Pi65* in *japonica* rice

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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 the rice blast resistant varieties is one of the best ways to control rice blast disease. Using a map-based cloning strategy, here, we cloned a novel rice blast resistance gene, *Pi65* from the resistant variety GangYu129 (abbreviated GY129, *O. sativa japonica*). Overexpression of *Pi65* in the susceptible variety LiaoXing1 (abbreviated LX1, *O. sativa japonica*) enhanced rice blast resistance, while knockout of *Pi65* in GY129 resulted in susceptible 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 *M. oryzae* collected from northeast China. There are sixteen amino acids differences between the *Pi65* resistance and susceptible alleles. Compared with the *Pi65* resistant allele, the susceptible allele deleted one LRR domain. *Pi65* was constitutively expressed in whole plants, and it could be induce expressed in the early stage of *M. oryzae* infection. Transcriptome analysis revealed that numerous genes associated with disease resistance were specifically upregulated in GY129 24-hour post inoculation (HPI), on the contrary, the photosynthesis-and carbohydrate metabolism-related genes were particularly downregulated 24 HPI, demonstrating that the disease resistance associated genes has been activated in GY129 (carrying*Pi65*) after rice blast fungal infection, and the cellular basal and energy metabolism was inhibited simultaneously. Our study provides genetic resources for improving rice blast resistance as well as enriches the study of rice blast resistance mechanisms.

Key words: Disease resistance, Gene cloning, *Japonica* rice, *M. oryzae*

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 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 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, although the substitution of genetic background can be achieved through multiple backcrosses, it is time consuming and difficult to apply in resistant breeding. 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) (Ronald and Beutler 2010; Cheng et al. 2012; Liu et al. 2013). PTI is a nonspecific defense response, which is characterized by

broad resistance spectrum and persistent resistance. The pathogen-related molecular modes that stimulate PTI, such as polysaccharides, polypeptides and flagellin, exist widely and conservatively in pathogens (or nonpathogen specific) (Ronald and Beutler 2010; Chen and Ronald 2011; Segonzac and Zipfel 2011); 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 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 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 both CRISPR/Cas9 mediated gene knockout mutation in resistant rice variety of GY129 and mutation mediated overexpression of *Pi65* in susceptible rice variety of LX1. 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 as well as 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 disease reactions of the GY129/LX1 BC_1F_2 population and *Pi65* knockout and overexpressed mutants. Donor variety GY129 is used in international research and breeding (Mukhina et al. 2020).

The rice plants were sown in black plastic containers (10 × 7.0 × 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 × 47 × 15 cm^3). The seedlings were grown in the greenhouse at 24 to 30 °C with an 8 h dark and 16 h light cycle until the three and half leaves stages (about three weeks old), then we did the spray-inoculation with spore suspensions (5 × 10^5 spores/mL) and placed in a blue box. 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, removed the sunshade and 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 the Calli of *Oryza sativa L. japonica* LX1 mediated by Agrobacterium tumefaciens. The transgenic plants were screened with a solution containing 300mg/mL carbenicillin and 50mg/mL hygromycin, and the hygromycin resistance gene was detected by PCR. All transgenic plants were properly managed in artificial climate incubator in Liaoning Province. More than 20 transgenic lines were obtained, and 3 independent T_2 lines were used in this study.

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 by Xiao et al. (Xiao et al. 2018). The organelle and GFP signals were detected with a Leica microscope (DM4000 B, Germany). The fusion of Arabidopsis aquaporin PIP2A with mCherry was used as a control for plasma membrane localization (Shaner et al. 2004; Nelson et al. 2007).

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 were 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 *japonica* rice, we tested *japonica* 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 Prime STAR® 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* was used as search strings in the nonredundant protein Rice Information GateWay (RIGW) database (https://rice.hzau.edu.cn/rice_rs3/) the top 24 high-similarity homologous genes have been downloaded for further analyses. Hmmer software was used for

identifying the LRR and kinase domain of each LRR-RLK genes. Clustal X was used for multisequence alignments (used kinase domains sequences and identity >50%), 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 process mediated by *Pi65*, we analyzed the *Pi65* associated genes' expression pattern. Total RNA was extracted from young seedlings of GY129 (carrying *Pi65* gene) and the *Pi65* knockout mutant KO-B 6 (abbreviated *Pi65*KO) 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 $\log_2$FC$\geqslant$2 and $\log_2$FC$\leqslant$-2 between two libraries.

Results

Molecular cloning and functional analysis of Pi65

In our previous work, *Pi65(t)* has been localized into a 430 Kb region between InDel-1 and SNP-4 on the short arm of chromosome 11 (Zheng et al. 2016). Within the *Pi65(t)* interval, we found four genes, Os11g0694500, Os11g0694600, Os11g0694850 and Os11g0695000, contain a typical LRR

domain. Sequence analysis result shown that, for Os11g0694500 and Os11g0695000, there were no sequence difference between the resistant parent GY129 and the susceptible parent LX1. However, the other two candidate genes, Os11g0694600 and Os11g0694850, sequence polymorphisms were presence 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 (Figs. 1a and S1a). We obtained a total of 12 and 4 independent T_0 CIRSPR knockout transgenic lines for 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 T_2 were selected for further rice blast resistance evaluation. The rice blast inoculation results shown that, in 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* was significantly larger than that 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 of LX1. The rice blast inoculation results indicated that, overexpression of *Pi65* in LX1 could let LX1 resistant to the virulent isolate of 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 gene contains 2 introns with lengths of 2923 bp and 386 bp, respectively. The full-length

cDNA of *Pi65* has a total of 3309 bp open reading frame (ORF), encoding 1102 amino acids (aa). The Pi65 protein sequence 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 the aa sites from 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, and the results revealed significant 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 fused the Pi65 coding region with a green fluorescent protein (GFP) and did the transient expression analysis to test its intracellular localization. Confocal laser scanning microscopy observation result shown that, when GFP was expressed alone, it localized to the whole rice cells (Fig. 3). However, both the membrane-specific marker of PIP2A (fused with mCherry) and GFP-Pi65 co-localized in the plasma membrane (Fig. 3). Indicating that Pi65 protein is a rice plasma membrane protein.

Pi65 phylogeny

The top 24 homologous genes of *Pi65* in (RIGW) genebank result suggests that the coupled genes could not have evolved from one another via a simple duplication event (Fig. S1b). The I indicates that the area is closely related to *Pi65*, the II indicates the second, the III indicates that the area is far away from *Pi65*, and the IV region indicates that the distance from *Pi65* is the farthest. The I contains

LOC_Os11g47210 and LOC_Os11g46980, LOC_Os11g47210 is the closest R gene to *Pi65* in plant phylogeny. Xa26 (LOC_Os11g47210) showed significant *Bacterial Blight resistance* in both seedling and booting stages of rice (Yang et al. 2003). LOC_Os11g46980 is involved in the response to elevated temperature and the LRR domain of this gene protein is important for the perception of elevated temperature. LOC_Os11g46980 may be partially overlapped with Xa3/XA26-mediated resistance pathways (Zhang et al. 2011). Thus, there is evidence that *Pi65* gene may trace their ancestry back to an ancient progenitor.

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 (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., *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 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 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 tested organs (Fig. 4a). Furthermore, to determine whether the expression of *Pi65* in GY129 could be induced expressed during *M. oryzae* infection or not, using the three and half-leaf stage-seedlings of GY129 (resistant variety) and LX1 (susceptible variety) as the materials, we did the rice blast inoculation and performed quantitative (q) RT-PCR analysis at six time points (0, 12, 24, 48, 72 and 96 HPI) (Fig. 4b). Compare to the control (mock treatment), after *M. oryzae* inoculation, at early stage (12h PHI), the expression level of *Pi65* was significantly increased in GY129, it reaching a peak at 72 HPI. However, in LX1, *pi65* (*Pi65*'s susceptible 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 for 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 the 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 2709 DEGs (619 upregulated genes and 2090 downregulated genes) and 2221 DEGs (299 upregulated genes and 1922 downregulated genes) were detected in GY129 and the KO*Pi65* mutant, respectively (Fig. 5a). Among all DEGs, 128 upregulated DEGs and 1399 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.

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that the pathways that were most highly enriched in common DEGs in GY129 and KO*Pi65* were associated with metabolic pathways, biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, Cutin, suberine and wax biosynthesis and fatty acid metabolites (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, although both GY129 and KO*Pi65* mutant rice presented resistance responses after inoculation with *M. oryzae,* however, the resistant responses in GY129 were much stronger than that in KO*Pi65*.

Additionally, 489 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), "response to stress" (GO:0006950), "response to salicylic acid"(GO:0009751) and "response to fungus" (GO:0009620), including 10 disease resistance protein (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 kinases, 4 putative disease resistance proteins, 1 receptor kinase-like proteins, 1 serine/threonine-protein phosphatases, 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 KOPi65 mutant, such as Bowman-Birk serine protease inhibitor (LOC_Os01g03330) and PPR repeat family (LOC_Os07g41810) genes (Fig. 6a, Tables S5). Overexpression of OsRSR1(LOC_Os11g12340) and OsRLCK5 can significantly improve the resistance of rice to sheath blight. OsRSR1 and OsRLCK5

can enhance the resistance of rice to sheath blight by regulating ROS balance through 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, 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). In conclusion, *Pi65* positively regulates many genes related to rice blast resistance.

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 pathways), carbohydrate metabolism (four pathways) and transport and catabolism (two pathways) (Fig. 6b). 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* (Tables 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 verified its was different from most NBS-LRR blast resistance genes in that it contained a typical kinase domain encoding a leucine-rich receptor protein kinase, Xa3/Xa26, which is similar to *Pi65*, may have originated 7.5 million years ago, prior to the differentiation of species with A and C genomes, so specific resistance to this seat has been conserved for a long time (Li et al. 2012), so the sequence of *Pi65* may also be evolutionarily conserved. 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, at the same time, the tertiary structure of proteins encoded by resistant and susceptible alleles is different, recognition of the effector can lead to a change in the conformation of NB-LRR protein, transforming NB-LRR from the inhibited state to the activated state, thus further activating downstream signal transduction (Collier and Moffett 2009). 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 be 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 1530 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 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". 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 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

Funding

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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

LW, WZ, DM, and ZM conceived and designed the experiments; MZ, SG, and CW prepared materials. LW conducted the experiment; LW, ZM, MZ, SG, ZM and CW acquired data; LW, ZM, HW, YB and GS analyzed and interpreted 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.

Figure legends

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 (Stu-dent's *t*-test; $*P< 0.05$, $***P < 0.001$, $****P < 0.0001$). c Blast reactions in GY129, 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 (Stu-dent's *t*-test; $*P < 0.05$).

Fig. 2 Structure of Pi65 and its deduced amino acid sequence. a LRRs and serine/threonine protein kinases are shown in CDS, respectively. 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.

Fig. 3 Intracellular localization of the Pi65 protein. Confocal fluorescence images and differential interference contrast (DIC) images of rice yellow tissue protoplasts in cells expressing GFP-Pi65 or GFP. The rice Pi65 protein fused with mCherry is a membrane marker. Scale bar: 5 μm.

Fig. 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 $-2^{\triangle\triangle}$CT method with Actin1 as an internal control. Data represent means with error bars showing ± s.d. (n=3) (Stu-dent's *t*-test; $*P< 0.05$, $**P < 0.01$, $***P < 0.001$).

Fig. 5 DEG analysis of GY129 and KO*Pi65*. a Venn diagram analysis of upregulated and downregulated

genes in GY129 and KO*Pi65* cells. b 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. c GO enrichment analysis for all DEGs in GY129 and KO*Pi65*, the X-axis represents the -log10 (Pvalue), and the left side of the Y-axis represents GO item types.

Fig. 6 GO enrichment analysis of GY129 of DEGs. a GO enrichment analysis of specifically up-DEGs in GY129, X-axis represents the -log10 (Pvalue), the left side of the Y-axis represents GO term types. b GO enrichment analysis of specifically down-DEGs with GY129, the X-axis represents the -log10 (Pvalue), the left side of the Y-axis represents GO term types.

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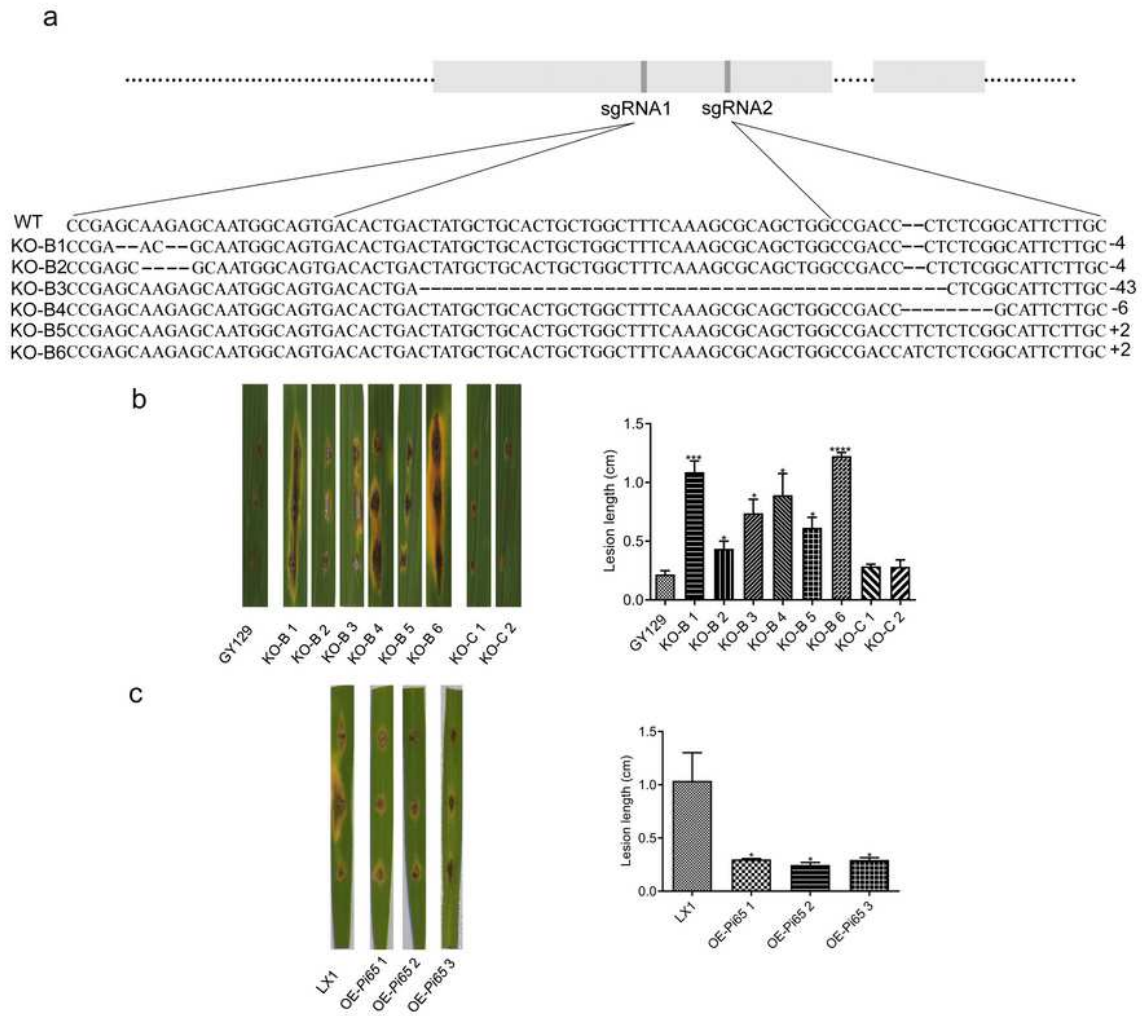
Figures



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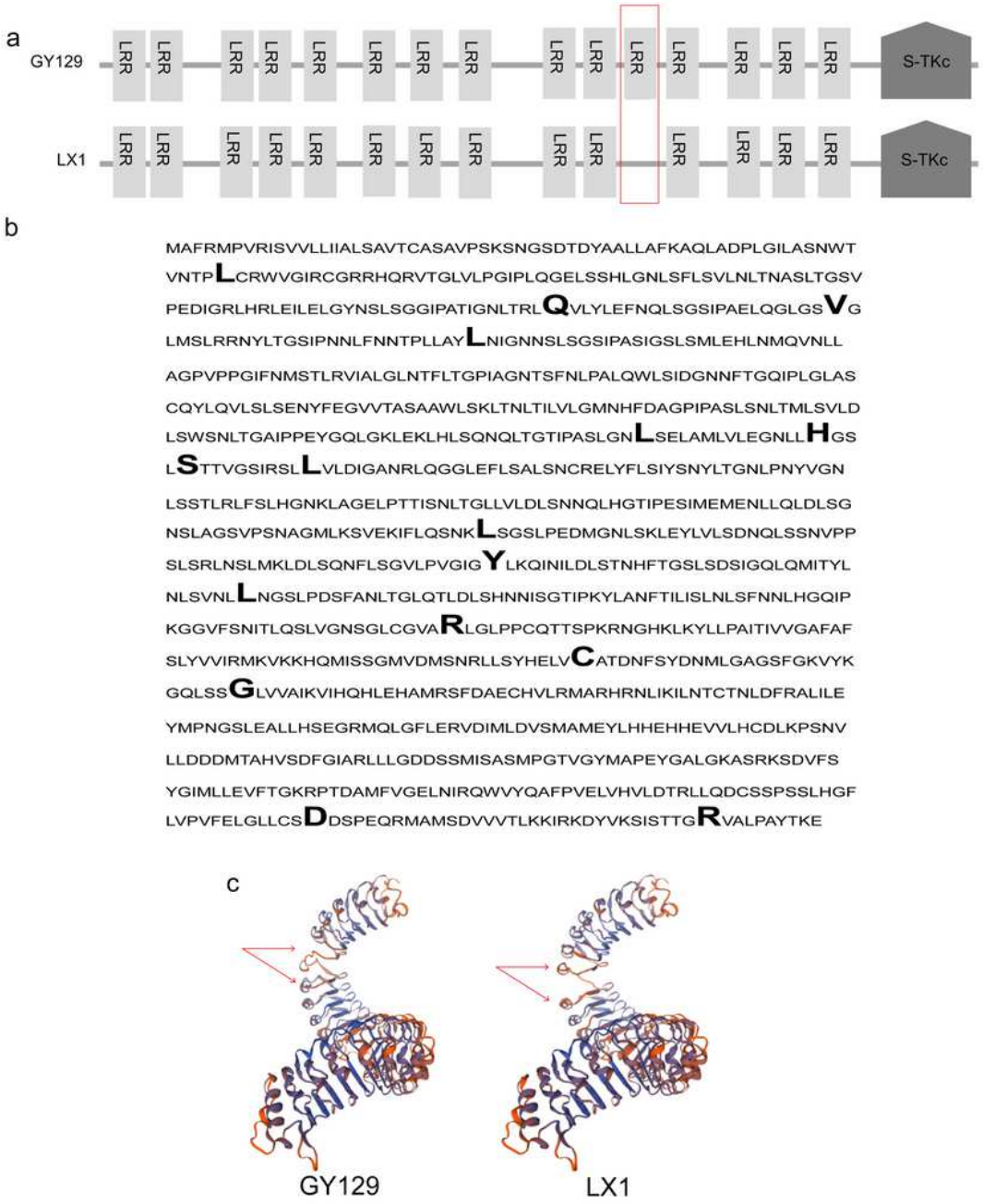

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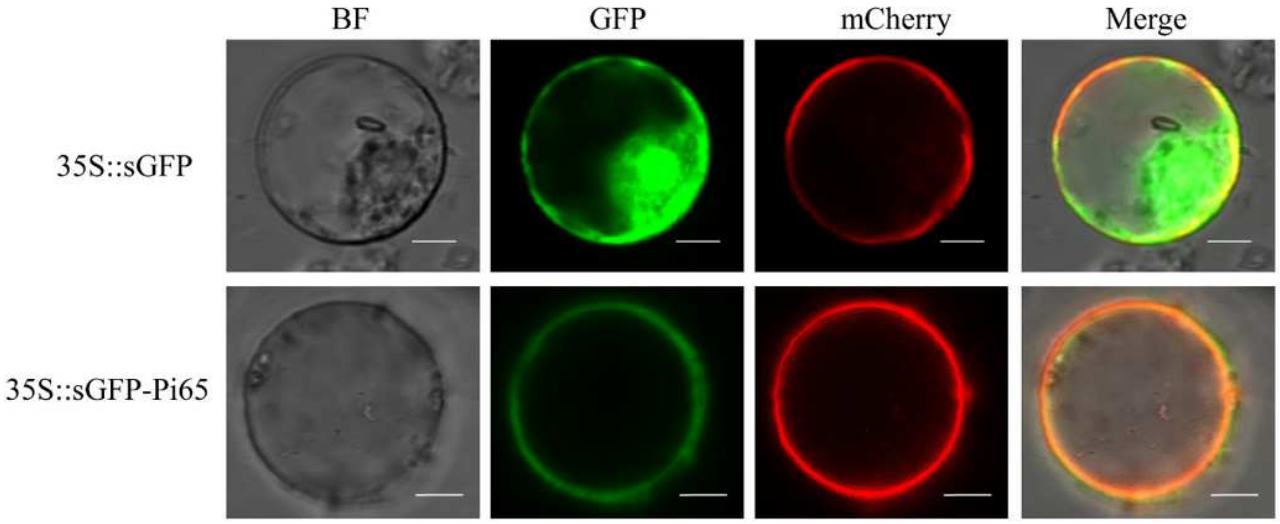

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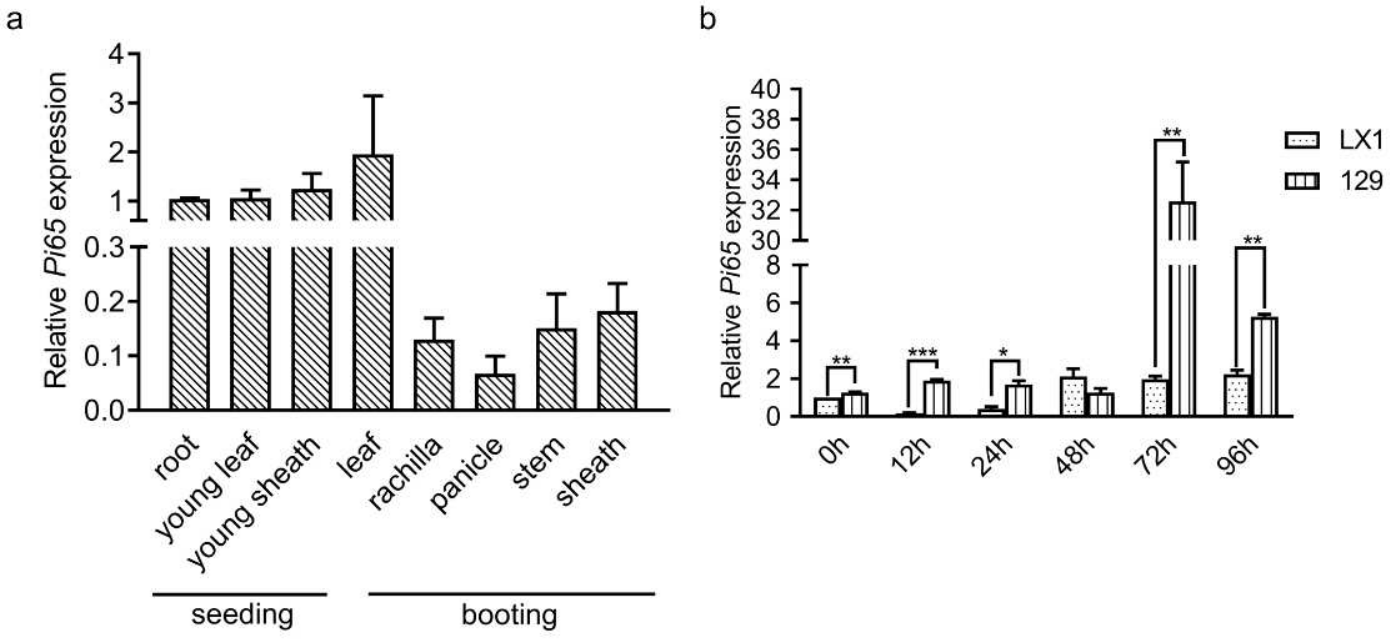

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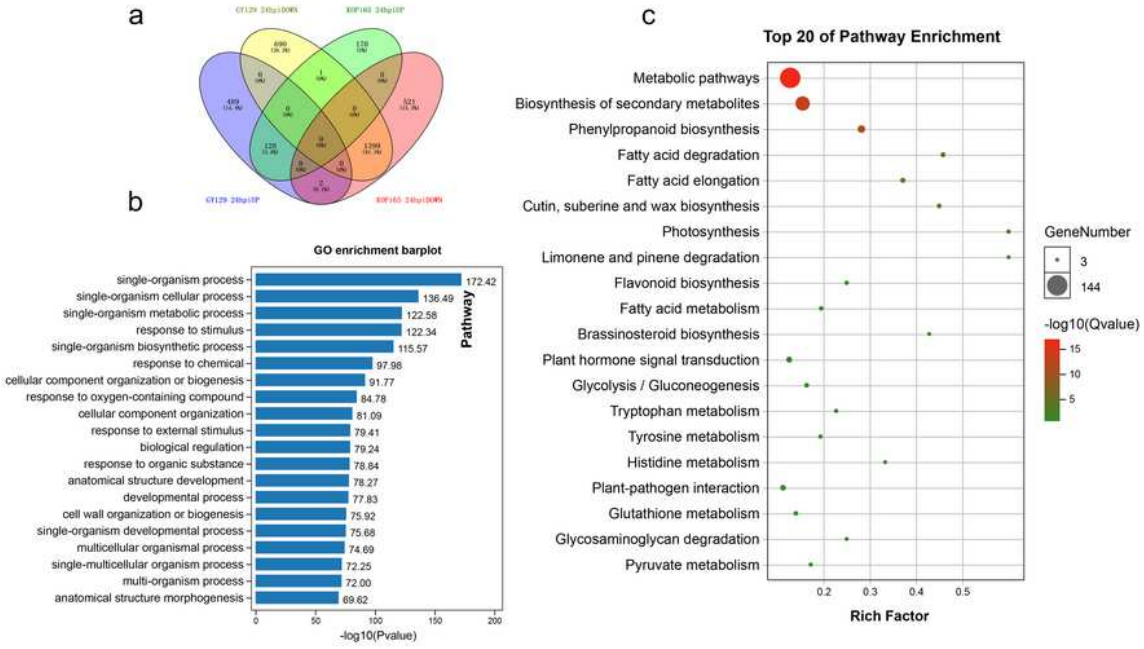

Figure 5

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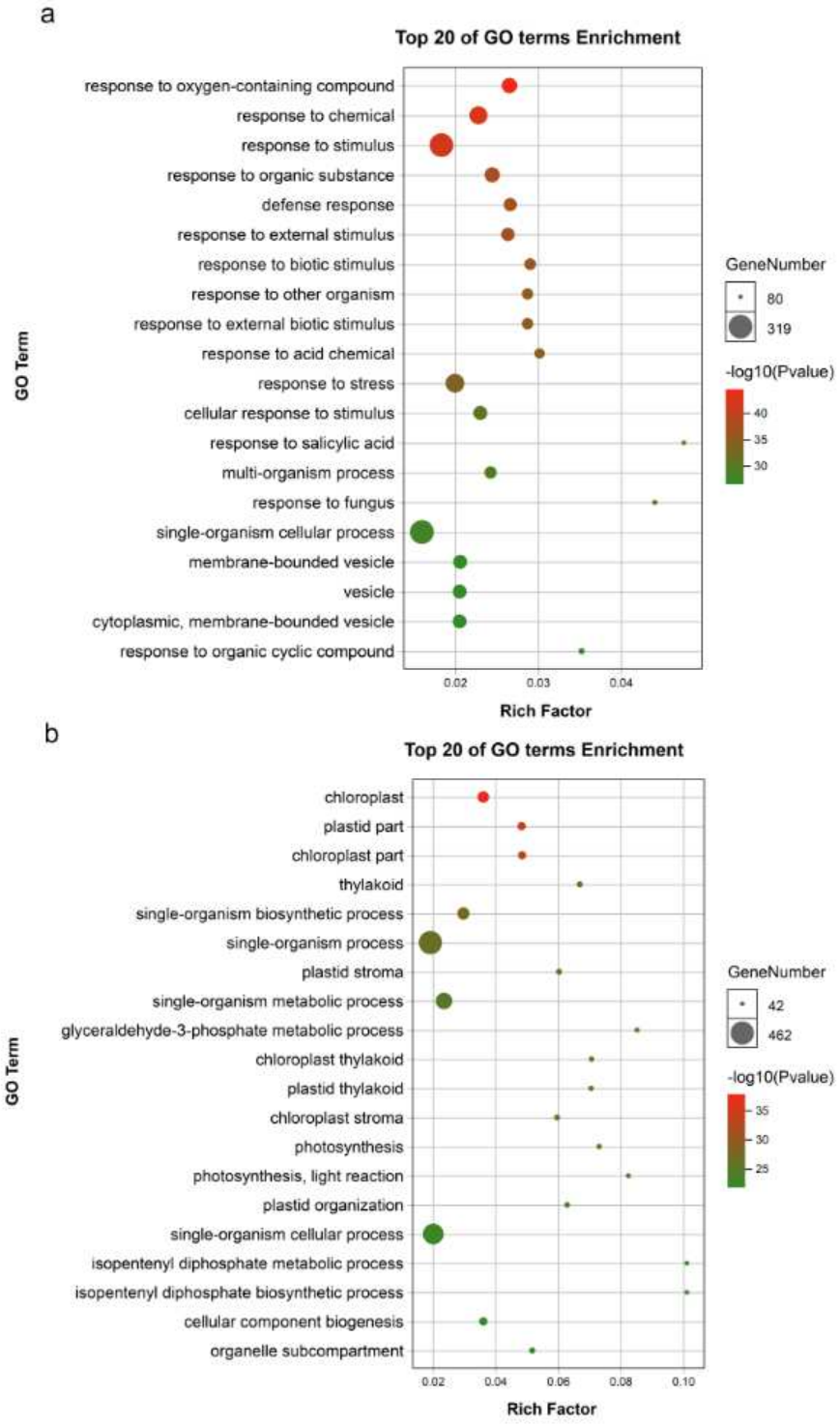

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