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Identification of *FERONIA-like receptor* genes involved in rice-*Magnaporthe oryzae* interaction

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Abstract

The receptor-like kinase (RLK) FERONIA functions in immunity in *Arabidopsis*. Here, we systemically screened rice *RLK* genes encoding FERONIA-like receptor (FLRs) that may be involved in rice-*Magnaporthe oryzae* interaction. The expression of 16 *FLR* genes was examined in response to the infection of *M. oryzae* in different rice varieties. For each *FLR* gene, at least two independent mutants were generated by CRISPR/Cas9 gene-editing technology in rice variety Zhonghua 11 (ZH11). Blast disease assay identified that the mutants of *FLR1* and *FLR13* showed increased susceptibility, whereas the mutants of *FLR2* and *FLR11* displayed enhanced resistance. Consistently, the mutant of *FLR1* enhanced, but the mutant of *FLR2* delayed the *M. oryzae* infection progress, which might be associated with the altered expression of defense-related genes. Together, these data indicate that at least 4 *FLR* genes are involved in rice-*M. oryzae* interaction and thus are potentially valuable in blast disease resistance.

Keywords: FERONIA-like receptors, Rice blast resistance, *Magnaporthe oryzae*, Receptor-like kinase, CRISPR/Cas9

Background

RLK proteins play vital roles in various biological processes including plant-microbe interactions. When a pathogenic microbe colonizes on the plant surface, the cell membrane-localized RLKs are employed to specifically recognize the cognate pathogen-associated molecular pattern (PAMP) via their extracellular domains to activate an innate immunity, termed PAMP-triggered immunity (PTI). For example, the *Arabidopsis* receptor protein FLAGELLIN-SENSING (FLS2) associates with the co-receptor protein BRASSINOSTEROID INSENSITIVE 1–SSOCIATED KINASE 1 (BAK1) in a complex to sense the bacterial PAMP molecule flg22 (Sun et al. 2013). Similarly, the lysin motif-containing receptor proteins LYP4, LYP6, CHITIN ELICITOR RECEPTOR KINASE (CERK1) and CHITIN ELICITOR BINDING PROTEIN (CEBiP) sense the fungal PAMP molecule chitin (Shimizu et al. 2010; Liu et al. 2012). This cognition activates the kinase activity of RLK to transduce the immune signal from the apoplast to the cytoplasm and the nucleus for initiating immune responses, such as the induction of defense-related genes, the activation of mitogen-associated protein kinase (MAPK), H_2O_2 accumulation, and callose deposition (Bigeard et al. 2015). The successful pathogens secret effector proteins to overcome PTI for pathogenesis (Tsuda and Katagiri 2010). In turn, plants employ resistance (R) proteins to recognize cognate effectors in classic gene-for-gene manner, leading to effector-triggered immunity (ETI).

In *Arabidopsis*, *Catharanthus roseus* RLK1-like kinase (*Cr*RLK1L) proteins play vital roles in regulating both PTI and ETI (Mang et al. 2017; Stegmann et al. 2017). For example, FERONIA (FER) is a well-characterized *Cr*RLK1L protein that suppresses resistance to powdery mildew (Kessler et al. 2010), whereas enhances resistance to *Pseudomonas syringae* tomato DC3000

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(Stegmann et al. 2017). FER acts as a scaffold that is required for the assembly of receptor complexes associated with the receptors FLS2, EF-TU RECEPTOR (EFR), and their co-receptor BAK1. Mutation in *FER* gene leads to the reduction of the ligand-induced assembly of immune receptor complexes FLS2-BAK1 and EFR-BAK1 (Stegmann et al. 2017). Moreover, the FER-scaffold can be inhibited by the binding of a small peptide, RAPID ALKALINIZATION FACTOR 23 (RALF23), which acts as a ligand associated with the extracellular Malectin domain of FER to facilitate infection. Consistently, the transgenic lines over-expressing RALF23 displayed enhanced susceptibility to *Pto* DC3000 (Stegmann et al. 2017). Another two *Cr*RLK1L proteins, ANXUR1 (ANX1) and ANX2, competitively associate with the co-receptor BAK1 to block the formation of FLS2-BAK1 complex, leading to suppression of FLS2-mediated PTI signaling. ANX1 also associates with RESISTANT TO PSEUDOMONAS SYRINGAE2 (RPS2) to facilitate its protein degradation, leading to compromised ETI responses (Mang et al. 2017).

The functions of *Cr*RLK1L proteins are conserved in cell growth, cell-cell communication and cell wall integrity (CWI) in *Arabidopsis* and rice (Lindner et al. 2012; Franck et al. 2018). For example, mutations in *Arabidopsis FER* lead to sterile and growth defection with semi-dwarf, abnormal trichomes, box-shaped epidermal cells, defected root hair (Duan et al. 2010; Li et al. 2015), which can be complemented by exogenous expression of rice *DWARF AND RUNTISH SPIKELET1* (*DRUS1*) gene (Pu et al. 2017). In rice, *DRUS1* and *DRUS2* are close homologous and confer functions in fertilization and growth similar to those of *FERs* in *Arabidopsis*. The *drus1* mutant has defection in male gametophyte development and *drus1drus2* double mutant has growth defection (Pu et al. 2017). *Ruptured Pollen tube* (*RUPO*), a rice ortholog of *Arabidopsis ANX1/2*, was conservatively required for pollen tube growth and integrity in rice (Liu et al. 2016). However, it is unclear whether rice *CrRLK1L* genes play roles in rice-*M. oryzae* interaction.

In rice, there are 16 genes annotated as members of the *CrRLK1L* gene family in the database of the MSU Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/), which are also named *FERONIA-like receptors* (*FLRs*) (Li et al. 2016). To identify which of these genes are involved in rice-*M. oryzae* interaction, we examined their expression upon the infection of *M. oryzae* in different rice accessions. We also generated mutants for each gene via CRISPR/Cas9 approach and applied them into blast disease assays. Our data indicate that multiple *FLR* genes are differentially responsive to *M. oryzae* infection and 4 of them may oppositely regulate rice blast disease resistance, thus provide a start point for further dissecting their functions in rice-*M. oryzae* interaction.

Results

FLR genes are differentially responsive to *M. oryzae* infection in different rice accessions

In rice, there are 16 FLR proteins which contain 841–955 amino acid residues (Li et al. 2016). They form five clades in a phylogenetic tree together with 17 homologous members from *Arabidopsis* (Additional file 1: Figure S1). FLR1/DRUS1 (DWARF AND RUNTISH SPIKELET 1) and FLR2/ DRUS2 are the closest orthologs to the *Arabidopsis* FER, whereas FLR6 is close to ANX1 and ANX2 (Additional file 1: Figure S1). To identify which of these 16 FLR protein-encoding genes are involved in rice-*M. oryzae* interaction, we first analyzed their expressions using RNA-seq data, which were derived from the leaves of a susceptible accession Lijiangxin Tuan Heigu (LTH) and four resistant accessions, including IRBLz5-CA, IRBL9-W, IRBLkm-Ts and YH2115 before and after inoculation with the *M. oryzae* strain Guy11. YH2115 is an elite restorer line for three-line hybrid rice (Shi et al. 2015). IRBLz5-CA, IRBL9-W and IRBLkm-Ts carry blast resistance gene *Pi-2*, *Pi-9* and *Pi-km*, respectively (Shi et al. 2015). Apart from *FLR6*, *FLR9*, *FLR10* and *FLR13* that were consistently expressed at the background level, the rest 12 *FLR* genes exhibited differential responses to *M. oryzae* infection in at least two accessions (Additional file 1: Figure S2). To confirm this observation, we examined their expression patterns in leaves after inoculation with the *M. oryzae* strain GZ8 by quantitative real-time polymerase chain reaction (qRT-PCR). Compared with mock inoculation, eight *FLR* genes showed differential response patterns between LTH and IRBLkm-Ts upon *M. oryzae* inoculation, including *FLR1*, *FLR2*, *FLR5*, *FLR9*, *FLR10*, *FLR11*, *FLR13* and *FLR15* (Fig. 1a), whereas the rest 8 *FLR* genes showed no significant difference (Fig. 1b). These data indicate that different *FLR* genes exhibit different responses in different rice accessions to *M. oryzae* infection.

FLR mutants were generated by CRISPR/Cas9 gene-editing technology

In order to confirm that the *M. oryzae*-responsive *FLR* genes are involved, whereas those that are not responsive to *M. oryzae* are not involved in rice-*M. oryzae* interaction, we generated knock-out mutants of these *FLR* genes (named *flr-c*) respectively by CRISPR/Cas9 gene-editing technology in rice cultivar Zhonghua 11 (ZH11) via designing the guide RNA to target the N-terminal sequence of each *FLR* gene (Additional file 1: Figure S3 and Table 1). Subsequently, we obtained at least two homozygous mutants for each of 13 *FLR* genes in T1 generation, and for *FLR5* and *FLR6* in T2 (Additional file 1: Figure S3 and S4). However, we failed to get homozygous mutants for *FLR9*/*RUPO* (*RUPTURED POLLEN TUBE*) (Additional file 1: Figure S4). This is consistent with a previous report that *RUPO* is critical for

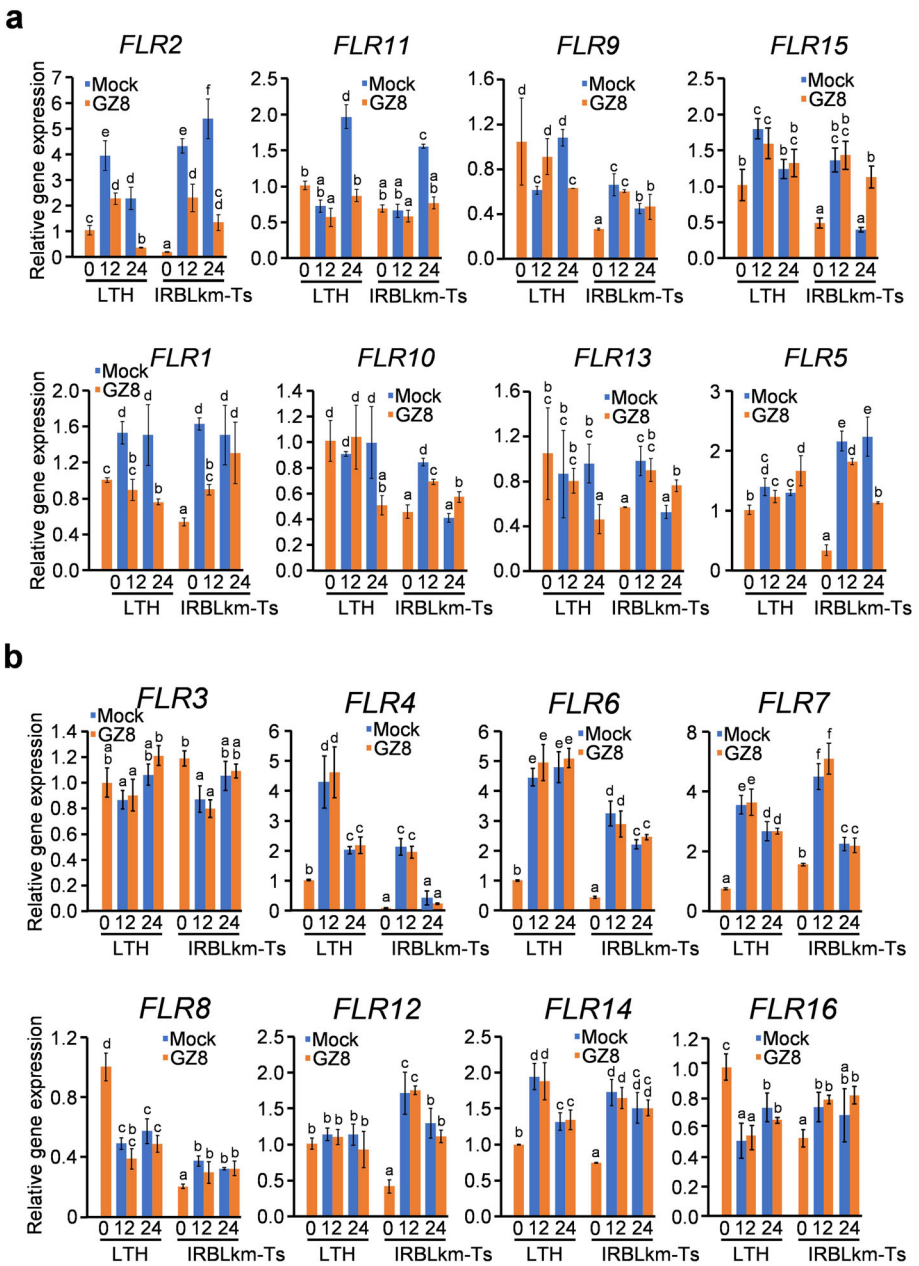

Fig. 1 Expression profiles of *FLR* genes in rice accessions LTH and IRBLkm-Ts upon *M. oryzae* inoculation. The seedlings of LTH and IRBLkm-Ts were spray-inoculated with *M. oryzae* strain GZ8, with ddH_2O treatment as mock inoculation. The samples harvested at 0, 12 and 24 hpi for investigating expression profiles of *FLR* genes. **a** *FLR* genes that are responsive to *M. oryzae* infection. The error bars indicate the mean ±SE (n=3). **b** *FLR* genes that are not responsive to *M. oryzae* infection

male gametophyte transmission and cannot generate homozygous knock-out mutant (Liu et al. 2016). To guarantee that the loss-of-function mutants were obtained, we chose those harboring insertion or deletion to generate a premature stop codon, leading to a large truncation for each *FLR* gene. For example, *flr1-c-1* contains a 2-bp deletion at the gRNA targeting site (Additional file 1: Figure S3), leading to a premature protein with amino acids change started at position 60 and truncated at position 247, whereas, *flr1-c-2* includes three types of 1-bp insertion at position 180 (Additional file 1: Figure S3), leading to a same premature protein which has only one amino acid insertion at position 60 in comparison with that in *flr1-c-1* (Additional file 1: Figure S4). Four *flr2-c* mutants have different mutation types that respectively carry 1- and 4-bp deletion, and 1-bp insertion at nucleotide position 277 at the gRNA targeting site (Additional file 1: Figure S3), leading to premature proteins with

Table 1 gRNA targets and genotyping primers for each mutant of *FLR* gene in rice

Gene ID	Name	gRNA targets	Genotyping primers
LOC_Os03g21540	*FLR1*	GGCAGGCCAACGACACGGA	FLR1-F: GTTTGTGGCCGTGCTTCTTC FLR1-R: GGAACGAGTAGGTGAAGGGC
LOC_Os01g56330	*FLR2*	TAGGGCACCTGCGGCACCG	FLR2-F: TCTTGCTCCTCCTCCTCCTC FLR2-R: TCTGGTAGGCGCTGAAGTTG
LOC_Os05g25450	*FLR3*	GACAGTGTGAGCCATTGGA	FLR3-F: CATTGCCTTTCACCGATGGC FLR3-R: TTGCCTCAAATCCCACCCAG
LOC_Os05g25370	*FLR4*	CGCTATGGCGATTGATAAG	FLR4-F: CATGTCCAAGCCCTACCCAG FLR4-R: ATGGTGCAAACGTGGAGCTA
LOC_Os05g25350	*FLR5*	GACAGTGCGACCCATTGGA	FLR5-F: TTACGCAATCGTTCCCACCT FLR5-R: TCTAGTGTCCCCCTCCCAAC
LOC_Os05g20150	*FLR6*	GGTGCCGTATATGACGGCG	FLR6-F: ATGCCGGTGGATGTAGACAC FLR6-R: ATCGACGGCAGAAAATGGCT
LOC_Os07g05370	*FLR7*	TAAGGAGGATCCTGGCTTG	FLR7-F: CTGAACTCTCCGTCGTCACC FLR7-R: TGTGTCTGGCAACTCGGTTT
LOC_Os04g52860	*FLR8*	TGTGGTTCAAACGCGACTG	FLR8-F: AAGAAACGCGCGGAATGGTA FLR8-R: TCTAGGGTGCAGATGCTTGC
LOC_Os06g03610	*FLR9*	TCGTCGCGGAAGATCCGGG	FLR9-F: ACGTTGATCCGGTACACCAC FLR9-R: GGCAAGGTGTACAAGACGGA
LOC_Os03g55210	*FLR10*	CAGTAATCTTGATGTCCAC	FLR10-F: TGACCAGGCTGGGAGATGAA FLR10-R: TGCCATTGCATCACAAGCAG
LOC_Os10g39010	*FLR11*	GGACTTTCGTCCCGGACGA	FLR11-F: GAAGGGGAAGAAGTGGAGGC FLR11-R: CTCTTCCTTGTCGTGGGCTT
LOC_Os03g03280	*FLR12*	AAGGACGAGTGCGATGCGG	FLR12-F: ATCAGCGTCCACTCCATTGT FLR12-R: GGATCGTGTTGGAGCCGGT
LOC_Os03g17300	*FLR13*	GAAGAGGTAGCTGTCCGCC	FLR13-F: CATGGTGGGGCAACCTACTC FLR13-R: GGTACCGGAAGGGGAAGAAG
LOC_Os01g06280	*FLR14*	CTTGAACACCCTGGCGCTC	FLR14-F: GCCTTGTTGCTGCATACACTC FLR14-R: AGCAGGAACTCCTCGCAC
LOC_Os06g22810	*FLR15*	GCAGGCTTGAATTGCGCTG	FLR15-F: AGCTTGCAATTGTAGCTGCC FLR15-R: GGATTTCCAGGGAGGTGTCC
LOC_Os05g06990	*FLR16*	CCACGCTGAATTCGGTGTC	FLR16-F: ACTGTGGTTCCACGGTCGAT FLR16-R: GGAATTGCTTGGCTGAGGGA

amino acid change started at position 93 and truncated at position 313 (*flr2-c-1*), 312 (*flr2-c-2*) or 243 (*flr2-c-3 and -4*), respectively (Additional file 1: Figure S4). Besides, we obtained two independent mutants with large truncations at the N-terminus for each of *FLR3*, *FLR4*, *FLR5*, *FLR6*, *FLR7*, *FLR8*, *FLR10*, *FLR12*, *FLR14*, *FLR15* and *FLR16*, three mutants for *FLR11*, and four mutants for *FLR13* (Additional file 1: Figure S3 and S4). These mutants are different from the previously reported ones in mutation site and genetic background (Li et al. 2016; Pu et al. 2017; Yang et al. 2020), and thus should be valuable in functional analysis of *FLR* genes.

Mutants of 4 *FLR* genes exhibit altered blast disease resistance phenotypes

To test whether single mutant of each *FLR* gene influences rice sensitivity to *M. oryzae*, all the mutants were subjected to blast disease assay by punch-inoculation with the *M. oryzae* strain GZ8 at the seedling stage. Compared with the disease symptoms on leaves of the wild-type (WT) ZH11, increased lesion size was observed on the leaves of *flr1-c-1, flr1-c-2, flr13-c-1 and flr13-c-2* (Fig. 2a, b). Consistently, these mutants supported significantly more fungal growth than their corresponding WT control at 7 days post-inoculation (dpi) (Fig. 2c). In contrast, decreased lesion size was observed on the infected leaves of *flr2-c-1, flr2-c-2, flr11-c-1 and flr11-c-2* (Fig. 2a, b), which supported significantly less fungal growth than control at 7 dpi (Fig. 2c). However, similar lesion size and fungal growth were observed on the infected leaves of the other *FLR* mutants and WT control (Fig. 2). Together, these observations indicate that *FLR1* and *FLR13* may positively, whereas *FLR2* and *FLR11* may negatively act in rice resistance to *M. oryzae.*

FLR1 and FLR2 act oppositely in rice resistance against *M. oryzae*

To confirm that *FLR1, FLR2, FLR11* and *FLR13* act in rice-*M. oryzae* interaction, we performed blast disease assay at adult stage by punch-inoculation with the *M.*

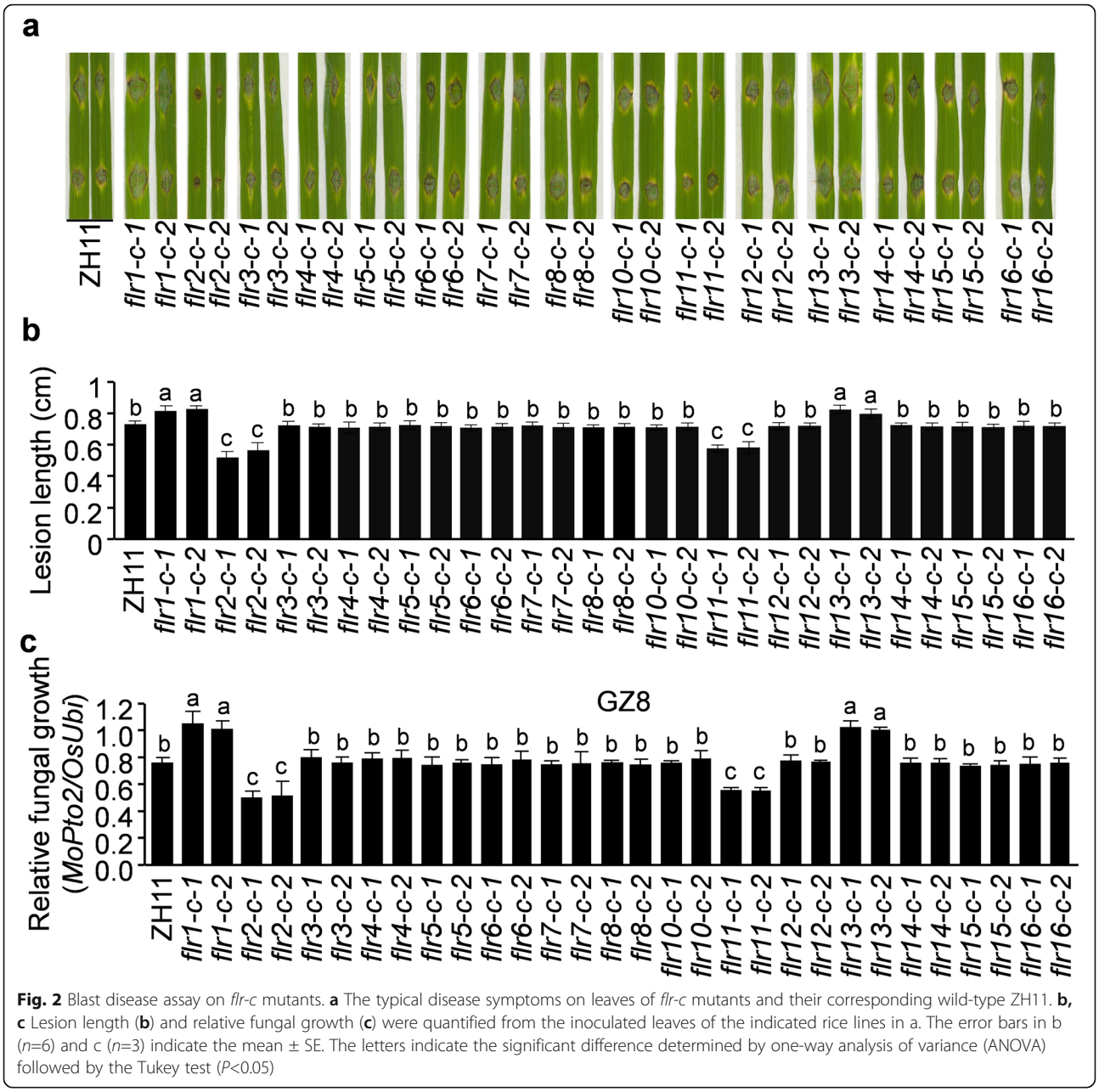

Fig. 2 Blast disease assay on *flr-c* mutants. **a** The typical disease symptoms on leaves of *flr-c* mutants and their corresponding wild-type ZH11. **b, c** Lesion length (**b**) and relative fungal growth (**c**) were quantified from the inoculated leaves of the indicated rice lines in a. The error bars in b (n=6) and c (n=3) indicate the mean ± SE. The letters indicate the significant difference determined by one-way analysis of variance (ANOVA) followed by the Tukey test (P<0.05)

oryzae strain GZ8. At the 2-month-old adult stage, punch-inoculation generated bigger disease lesions in mutants of *FLR1* and *FLR13* than that in WT (Fig. 3a, b), but smaller disease lesions in mutants of *FLR2* and *FLR11* (Fig. 3a, b). These data indicate that *FLR1, FLR2, FLR11* and *FLR13* act consistently at both adult and seedling stages.

FLR1 and *FLR2* are the closest orthologs to the Arabidopsis *FER* that regulates vegetative growth and immunity (Deslauriers and Larsen 2010; Stegmann et al. 2017). To explore why they act oppositely in rice-*M. oryzae* interaction, we compared the infection process of *M. oryzae* in the mutants *flr1-c-1* and *flr2-c-1* with that in WT. The leaf sheaths were detached from *flr1-c-1, flr2-c-1* and WT at five-leaf seedling stage and inoculated with a GFP-tagged *M. oryzae* strain GZ8. Under fluorescence microscope, quantification analysis indicated that around 25% spores formed invasive hyphae in the primary infected cells (the first cells) in WT, 55% spores formed invasive hyphae in the first cells and 5% had already expanded to the second cells (the closest neighbor cells of the first cells) in *flr1-c-1*, but only 10% spores formed invasive hyphae in the first cells in *flr2-c-1* at 24 hours post-inoculation (hpi) (Fig. 3c, d). The infection progress was further enhanced in *flr1-c-1* at 48 hpi with 50% spores forming invasive hyphae in the third

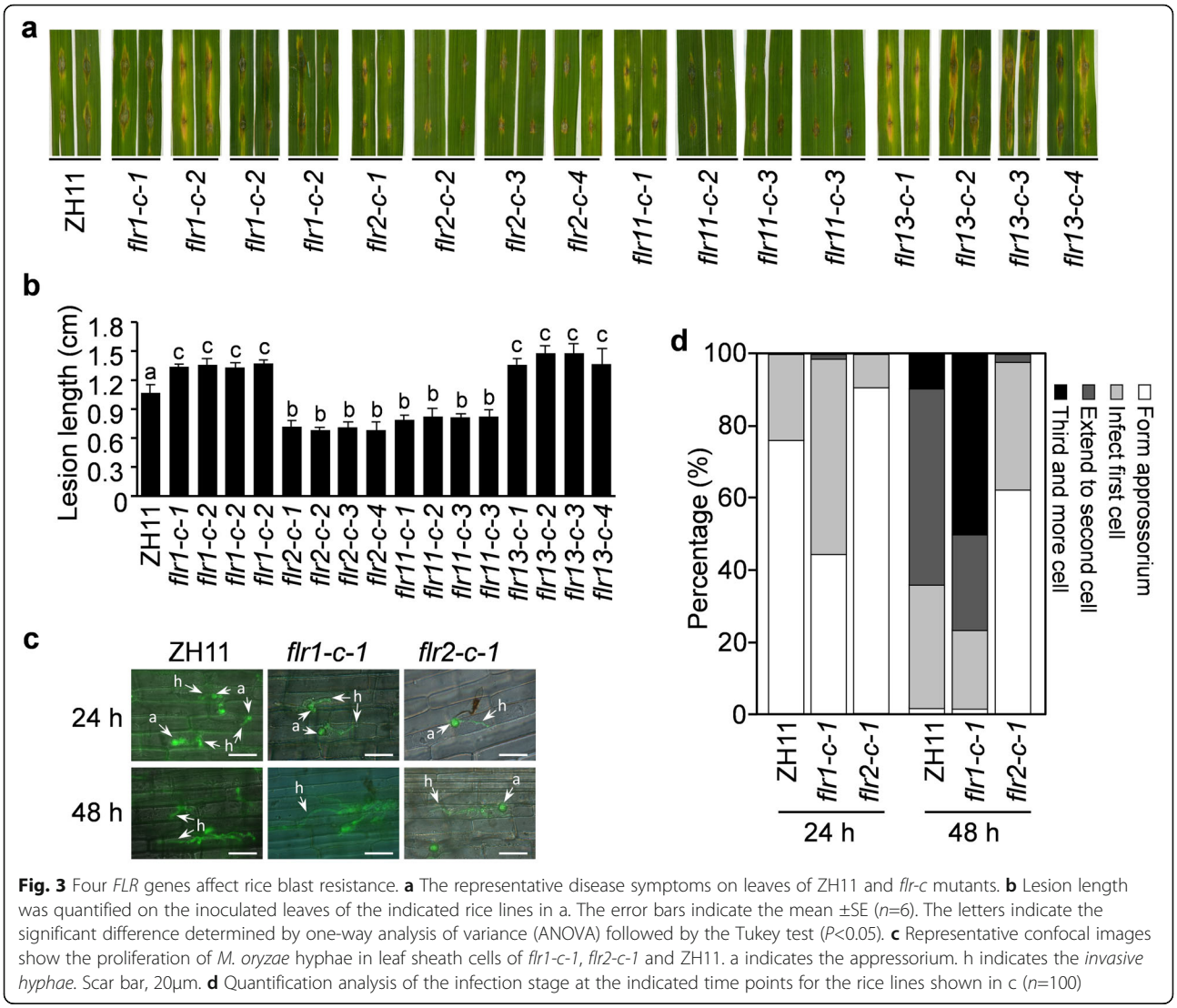

Fig. 3 Four *FLR* genes affect rice blast resistance. **a** The representative disease symptoms on leaves of ZH11 and *flr-c* mutants. **b** Lesion length was quantified on the inoculated leaves of the indicated rice lines in a. The error bars indicate the mean ±SE (n=6). The letters indicate the significant difference determined by one-way analysis of variance (ANOVA) followed by the Tukey test (P<0.05). **c** Representative confocal images show the proliferation of *M. oryzae* hyphae in leaf sheath cells of *flr1-c-1*, *flr2-c-1* and ZH11. a indicates the appressorium. h indicates the *invasive hyphae*. Scar bar, 20μm. **d** Quantification analysis of the infection stage at the indicated time points for the rice lines shown in c (n=100)

(the second closest neighbors of the first cells) and more cells compared with only 10% in WT. In contrast, only 3% expanded to the second cells and no hyphae in the third and more cells in *flr2-c-1* at 48 hpi (Fig. 3c, d). These observations indicate that the infection process of *M. oryzae* is facilitated in *flr1-c-1*, but delayed in *flr2-c-1*. Therefore, *FLR1* and *FLR2* act oppositely in rice resistance against *M. oryzae*.

To confirm the roles of *FLR1* and *FLR2* in rice defense against *M. oryzae*, we examined the expression of several defense-related genes in spray-inoculated seedlings of *flr1-c-1, flr1-c-2, flr2-c-1* and *flr2-c-2* in comparison with that of WT. Disease symptoms and fungal growth were shown at 5 dpi of the blast fungus (Fig. 4a, b). The expression of three defense-related genes was examined at 24 hpi, including *OsNAC4, PBZ1* and *PR10b*, which are often used as immune markers. *OsNAC4* encodes a transcription factor that positively regulates hypersensitive response (HR) cell death (Kaneda et al. 2009). *PBZ1* is a *PR10* family gene associated with cell death (Kim et al. 2011). *PR10b* was induced by *M. oryzae* infection (McGee et al. 2001) and often used as a marker to monitor rice immune response (Zhao et al. 2019). *OsNAC4, PBZ1* and *PR10b* were up-regulated in all tested lines at 24 hpi with the *M. oryzae* strain GZ8 compared with that in mock inoculated samples (Fig. 4c-e). However, their expressions were lower in *flr1-c-1,* but higher in *flr2-c-1* than in ZH11 (Fig. 4c-e), indicating that *flr1-c-1* is more susceptible, and *flr2-c-1* is more resistant to *M. oryzae* than ZH11.

Together, these data indicate that *FLR1* positively, but *FLR2* negatively act in resistance to *M. oryzae*.

Discussion

In this study, we systemically screened rice *FLR* genes to identify those that may play roles in rice-*M. oryzae* interaction. RNA-seq data showed that 12 out of 16 *FLR*

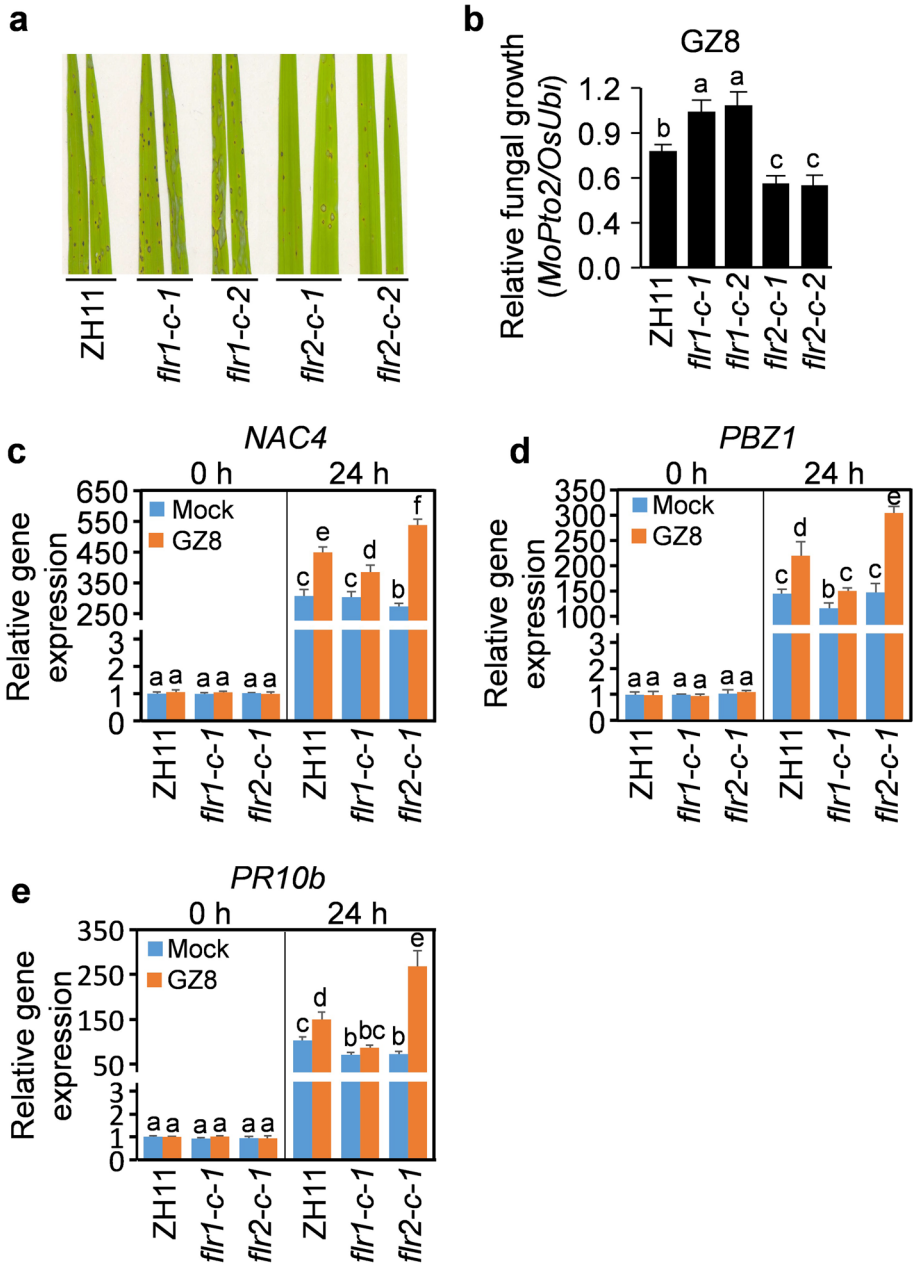

Fig. 4 *FLR1* and *FLR2* oppositely act in rice blast resistance. **a** Spray-inoculated disease symptoms of rice blast disease on the indicated rice lines. **b** Relative fungal growth was quantified on the inoculated leaves of the indicated rice lines in a. Expression of *NAC4* (**c**), *PBZ1* (**d**) and *PR10b* (**e**) were measured in *flr1-c-1*, *flr2-c-1* and ZH11 at 0 and 24 hpi and normalized by *OsUbi*. The error bars indicate the mean ±SE (n=3). The letters indicate the significant difference determined by one-way analysis of variance (ANOVA) followed by the Tukey test (P<0.05)

genes were differentially expressed among five rice accessions upon *M. oryzae* infection (Additional file 1: Figure S2), and of these, eight were confirmed to be differentially responsive to infection of *M. oryzae* between a susceptible and a resistant rice accession (Fig. 1). More than 30 mutants were subsequently generated via CRISPR/Cas9 gene-editing technology, which included at least two independent mutants for each of 15 *FLR* genes (Fig. 2 and Additional file 1: Figure S4). Mutants of 4 *FLR* genes displayed altered sensitivity to *M. oryzae*, of which *flr1-c-1*, *flr1-c-2*, *flr13-c-1* and *flr13-c-2* showed increased, whereas *flr2-c-1*, *flr2-c-2*, *flr11-c-1* and *flr11-c-2* showed decreased sensitivity (Fig. 2, Fig. 3 and Fig. 4). Our data are consistent with a recent report on the roles of 14 *FLR* genes in rice-*M. oryzae* interaction with a background of Nipponbare (Yang et al. 2020). Therefore, at least 4 *FLR* genes were identified to be involved in rice-*M. oryzae* interaction, of which *FLR1* and *FLR13*

positively, whereas *FLR2* and *FLR11* negatively act in rice blast disease resistance.

Through gene expression analysis and rice blast resistance investigation, we found some *FLR* genes that are responsive to *M. oryzae* infection don't affect disease symptom. However, we still can't completely rule out the possibility that these *FLR* genes act in rice blast disease resistance. One reason could be that gene expression might be age-dependent or rice accession-specific. This can explain the difference between RNA-seq and qRT-PCR data on detection of *M. oryzae*-responsive genes. Since there are no different disease phenotypes between the mutants of *M. oryzae*-responsive genes and their corresponding wild-type strain, these genes may play a subtle role in response to *M. oryzae*. Alternatively, since these genes are phylogenetically closely related, they may act redundantly, and single mutant could not exhibit obvious phenotype. In the phylogenetic tree, *FLR3* is closely related with *FLR4* and *FLR5*, *FLR7* with *FLR8*, and *FLR14* with *FLR15* and *FLR16* (Additional file 1: Figure S1). Functional redundancy is common in closely related genes. For example, *Arabidopsis ANX1* is closely related with *ANX2*, and they function redundantly to maintain cell wall integrity of pollen tube before reaching the female gametophytes (Boisson-Dernier et al. 2009; Miyazaki et al. 2009). Rice *FLR1*/*DRUS1* and *FLR2*/DRUS2 also have redundant functions in reproductive growth and development (Pu et al. 2017), although they act oppositely in response to *M. oryzae* (Fig. 3 and Fig. 4). Therefore, future work should construct double and/or triple mutants to exclude the involvement of other *FLR* genes in rice-*M. oryzae* interaction. It is intriguing that *FLR1* and *FLR2* act oppositely in rice-*M. oryzae* interaction because both are the closest orthologs to the *Arabidopsis FER* and are in the same clade (Additional file 1: Figure S1). This may be a common phenomenon existing in some homologous proteins that are involved in plant immunity. In *Arabidopsis*, three *Cr*RLK1L proteins, FER, ANX1 and ANX2, play antagonistic roles in immunity against bacterium pathogens, although they are in the same clade (Additional file 1: Figure S1). Compared with its corresponding WT, *fer* mutants showed increased susceptibility (Stegmann et al. 2017), but *anx* mutants showed enhanced resistance to *Pst* DC3000 (Mang et al. 2017). FER functions as a scaffold protein to facilitate, but ANX1 and ANX2 function as competitors to interfere with the formation of ligand-induced receptor complex (Franck et al. 2018). The similar phenomenon has also been reported for some homologous resistance proteins. For example, rice Pigm-R and Pigm-S, two homologous proteins encoded by two genes clustered at the *Pigm* locus, act antagonistically in blast disease resistance and yield traits (Deng et al. 2017). *Pigm-S* specifically expressed in panicle promotes seed setting and compensates the yield decrease caused by *Pigm-R*. Ectopic expression of *Pigm-S* competitively associates with *Pigm-R*, suppressing the formation of the Pigm-R homodimer to attenuate disease resistance. These results uncovered that the proper-spatiotemporal gene expression facilitates the balance between plant growth and resistance (Deng et al. 2017). Yang et al. (2020) recently reported that the expression of 4 *FLR* genes exhibited tissue specificity (Yang et al. 2020), implying that spatiotemporal gene expression may be employed by *FLR*s for properly performing their function. Therefore, one focus of the future research should be on how different *FLR* genes are spatiotemporally regulated upon the infection of *M. oryzae*.

It is a challenging question whether rice FLR receptors recognize RALF peptides to regulate rice-*M. oryzae* interaction, although RALF peptides have been characterized as the ligand for *Cr*RLK1L proteins to modulate plant growth and immunity (Stegmann et al. 2017; Zhong and Qu 2019). RALF peptides exist in both plants and pathogenic microbes and act as signal peptides for regulating plant growth, and plant-microbe interactions (Masachis et al. 2016; Stegmann et al. 2017). For example, a RALF from *Fusarium oxysporum* binds FER to regulate immune signal for pathogenesis (Masachis et al. 2016). The RALF orthologs widely exist in 26 species of phytopathogenic fungi (Thynne et al. 2017). We examined the genome of three *M. oryzae* strains (i.e. 70–15, Y34 and P131), but failed to identify any RALF orthologs. Therefore, *M. oryzae* may not exploit RALF to facilitate pathogenesis. Instead, other signal peptides secreted by *M.oryzae* or rice endogenous RALF peptides may act as the FLR's ligands to activate signal transductions. According to this speculation, the future work will focus on identification of RALFs or other potential ligand proteins that associate with different FLRs and dissection of their roles in rice-*M. oryzae* interaction.

Conclusions

Out of 16 *Cr*RLK1L family genes that are named *FLR*s, we identified 8 genes that are differentially responsive to *M. oryzae* infection. After generating mutants for 15 *FLR* genes and applying them in blast disease assays, we identified that 4 of them act in rice-*M. oryzae* interaction. Thus, our data provide a start point to dissect the roles of *FLR*s in the regulation of rice immunity against *M. oryzae*.

Methods

Plant materials and growth conditions

The rice accessions include two *Oryza sativa* L. ssp. japonica varieties, i.e. LTH and ZH11, and four *O. sativa* L. ssp. indica varieties, i.e. IRBLz5-CA, IRBL9-W, IRBLkm-Ts and YH2115. Mutants were generated from

the ZH11 background using CRISPR/Cas9 gene-editing technology. Rice plants were grown in the paddy field in Wenjiang District, Chengdu City, Sichuan Province, China during summer and grown in the paddy field in Lingshui County, Hainan Province, China during winter. The rice seedlings used in this study were grown in a greenhouse with a temperature of 28 ± 2 °C, relative humidity of 70%, and photoperiod of 12h light/12h dark.

Generation of mutants

The guide RNAs (gRNAs) targeting each *FLR* gene were selected using the E-CRISP Design Tool (http://www.e-crisp.org) (Heigwer et al. 2014). The primers were designed following the user manual of the *EXclone* cloning vector (Biogle Co., Ltd). The primer pair was aligned to form a short fragment with sticky ends, which was directly ligated with EX-Vector to generate the constructs of EX-Vector-FLR-1 to -16. The transgenic rice lines were generated from the ZH11 background by *Agrobacterium*-mediated transformation as described previously (Toki 1997). The transgenic lines were firstly screened on MS medium with 50 μg/L hygromycin. The positive lines were genotyped by primer pair across the gRNA target site. The PCR products were analyzed by Sanger sequencing to identify the positive transgenic plants with a null mutation at the gRNA target site.

Rice blast disease assay

The GFP-tagged *M. oryzae* strain GZ8 was used in this study for rice blast disease assay and infection process investigation. The strain was cultured for 10 days on oatmeal and tomato media (OTA) in a growth chamber with a temperature of 28 °C and a photoperiod of 12 h light/12 h dark for hyphae growth. At 10 days later, the hyphae were scratched and the cultures were then incubated for sporulation at 28 °C under 24 h continuous light condition. The spores were harvested with ddH_2O 4 days later and purified through nylon mesh. Finally, the spore concentration was adjusted to 5×10^5 conidia/mL for drop- and spray-inoculation.

For disease assay, six detached leaves from each rice line were punch-inoculated with *M. oryzae* following a previous study (Park et al. 2012). The biggest lesion in each leaf was measured by the software ImageJ for lesion length quantification. The relative fungal growth was indicated by the DNA amount ratio of *M. oryzae Pot2* to rice *OsUbi*. measured by quantitative reverse transcription PCR (qRT-PCR) (Li et al. 2019). The primers of MoPot2 RT-F: ACGACCCGTCTTTACTTATTTGG, MoPot2 RT-R: AAGTAGCGTTGGTTTTGTTGGAT and Ubi RT-F: GCCCAAGAAGAAGATCAAGAAC, Ubi RT-R: AGATAACAACGGAAGCATAAAAGTC were used for qRT-PCR to quantify DNA amount of *M. oryzae Pot2* and *OsUbi*, respectively. Three independent repeats are performed in each assay.

The leaf sheaths were detached from 1-month-old seedlings for investigation of *M. oryzae* infection process. The infection process was observed and recorded at 24 and 48 hpi under a laser scanning confocal microscope (LSCM). 100 single-spore penetration sites were recorded for calculating the infection process in each rice line.

RNA isolation and gene expression analysis

One-month-old seedlings were spray-inoculated with the *M. oryzae* strain GZ8 (1×10^5 conidia/mL) or ddH_2O (mock inoculation). The samples were harvested at 0, 12 and 24 hpi. Total RNA isolation and cDNA reverse transcription were performed following the manufacturer's instructions of TRIzol reagent (Invitrogen) and PrimeScript™ RT reagent Kit, respectively. SYBR Green mix (QuantiNova SYBR Green PCR Kit, Qiagen) was used for the qRT-PCR assay with the primers of OsNAC4 RT-F: TCCTGCCACCATTCTGAGATG and OsNAC4 RT-R: TTGCAGAATCATGCTTGCCAG, *OsPR10b*-RT-F*:* AAC ACGTGTGGTGGCACGTG and *OsPR10b*-RT-R*:* TCA TCTTGAGCATGCCGAAG, *OsPBZ1*-RT-F*:* ACACCA TGAAGCTTAACCCTGG and *OsPBZ1*-RT-R*:* TCGAGT GTGACTTGAGCTTCC, Ubi RT-F: GCCCAAGAAG AAGATCAAGAAC and Ubi RT-R: AGATAACAAC GGAAGCATAAAAGTC to investigate the expression profiles of *NAC4*, *PR10b*, *PBZ1* and *OsUbi*, respectively. The rice *Ubiquitin* (*OsUbi*) gene was used as an internal reference.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s42483-020-00052-z.

Additional file 1: Figure S1. A phylogenetic tree of *Cr*RLK1L family proteins. The full-length protein sequences were aligned by ClustalW and then generated the phylogenetic tree by the UPGMA method with 1000 bootstrap replicates in MEGA-X. This phylogenetic tree contains 33 *Cr*RLK1L family members in *Arabidopsis* and rice. In rice, the *Cr*RLK1L-encoding genes were named as *FERONIA-like receptors* (*FLRs*). **Figure S2.** RNA-seq heatmap of *FLR* genes in LTH and four resistant rice accessions upon *M. oryzae* infection. The 4-week-old seedlings of the indicated rice accessions were spray-inoculated with the *M. oryzae* strain Guy11. The samples were harvested for RNA-seq analysis at 0, 12, 24 hpi. **Figure S3.** DNA sequence of each *flr-c* mutant. The DNA sequence of the indicated homozygous mutants was identified by sequencing. The schematic diagram indicates the structure of *FLR* gene. The blue box indicates gene exon. The red triangle indicates the gRNA target site which corresponds to the red portion of the sequence below the schematic diagram. The black letters in red portion indicate inserted bases. The black dotted line fills up the gap caused by base deletion. The red dotted line fills up the gap between two adjacent bases without deletion. **Figure S4.** The truncated peptides encoded by mutated *FLR* genes. The proteins encoded by wild-type genes from ZH11 and mutated *FLR* genes were aligned by online multiple alignment tool (http://multalin.toulouse.inra.fr/multalin/). Red portion indicates the same protein sequence for both ZH11 and mutants. The blue or black portion indicates the mutated sequence after the mutation site in *flr-c* mutants. The black star indicates the ending site of protein encoded by mutated *FLR* gene.

Abbreviations

ANX: ANXUR; BAK1: BRASSINOSTEROID INSENSITIVE 1–ASSOCIATED KINASE 1; *Cr*RLK1L: *Catharanthus roseus* RLK1-like kinase; DRUS1: DWARF AND RUNTISH SPIKELET 1; EFR: EF-TU RECEPTOR; ETI: Effector-triggered immunity; FER: FERONIA; FLR: FERONIA-like receptor; *flr-c*: Knock-out mutants of *FLR* genes generated by CRISPR/Cas9 gene-editing technology; FLS2: FLAGELLIN-SENSING; gRNA: Guide RNA; HR: Hypersensitive response; LSCM: Laser scanning confocal microscope; LTH: Lijiangxin Tuan Heigu; LYPs: LYSIN MOTIF-CONTAINING PROTEINS; *M. oryzae*: *Magnaporthe oryzae*; MAPK: Mitogen-associated protein kinase; CEBiP: CHITIN ELICITOR BINDING PROTEIN; OsCERK1: CHITIN ELICITOR RRECEPTOR KINASE 1; *OsUbi*: Rice *ubiquitin*; OTA: Oatmeal and tomato media; PAMPs: Pathogen-associated molecular patterns; PTI: PAMP-triggered immunity; R protein: Resistance protein; RALF23: RAPID ALKALINIZATION FACTOR 23; RLK: Receptor-like kinase; RPS2: RESISTANT TO PSEUDOMONAS SYRINGAE 2; RT-PCR: Real-time fluorescent quantitative polymerase chain reaction; RUPO: RUPTURED POLLEN TUBE; ZH11: Zhonghua11

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Authors' contributions

Y-YH and W-MW conceived the project. Y-YH and X-XL designed and performed most of the experiments with support from YX, X-YL, Z-JH, L-FW, W-QD, L-LZ, J-QZ J-WZ, YL and JF. Y-YH, X-XL, YX, X-YL, HW, YZ, HF and MP grew the plants and harvested the samples. Y-YH, X-XL and W-MW wrote the manuscript. Y-YH, X-XL contributed equally to this work. The author(s) read and approved the final manuscript.

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Availability of data and materials

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

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