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The RLCK subfamily VII-4 controls pattern-triggered immunity and basal resistance to bacterial and fungal pathogens in rice

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SUMMARY

Receptor-like cytoplasmic kinases (RLCKs) mediate the intracellular signaling downstream of pattern-recognition receptors (PRRs). Several RLCKs from subfamily VII of rice (*Oryza sativa*) have important roles in plant immunity, but the role of RLCK VII-4 in pattern-triggered immune (PTI) signaling and resistance to pathogens has not yet been investigated. Here, we generated by multiplex clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9-mediated genome editing rice sextuple mutant lines where the entire RLCK VII-4 subfamily is inactivated and then analyzed the resulting lines for their response to chitin and flg22 and for their immunity to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Magnaporthe oryzae*. Analysis of the *rlckvii-4* mutants revealed that they have an impaired reactive oxygen system burst and reduced defense gene expression in response to flg22 and chitin. This indicates that members of the rice RLCK VII-4 subfamily are required for immune signaling downstream of multiple PRRs. Furthermore, we found that the rice RLCK VII-4 subfamily is important for chitin-induced callose deposition and mitogen-activated protein kinase activation and that it is crucial for basal resistance against *Xoo* and *M. oryzae* pathogens. This establishes that the RLCK VII-4 subfamily has critical functions in the regulation of multiple PTI pathways in rice and opens the way for deciphering the precise role of its members in the control of rice PTI.

Keywords: CRISPR/Cas9, multiplex genome editing, *Magnaporthe oryzae*, *Oryza sativa*, PTI, plant immunity, RLCK, rice, *Xoo*.

INTRODUCTION

Pattern-triggered immunity (PTI) is the first layer of the plant immune system that protects plants from infection by the majority of the potentially pathogenic microorganisms they encounter during their development. PTI is activated upon the detection of pathogen-associated molecular patterns (PAMPs) characteristic of broad classes of microbial organisms, such as flagellin (flg22) and peptidoglycan (PGN) from bacteria, or chitin from fungi (Boller & Felix, 2009; Kumar et al., 2021).

PAMPs are recognized by cell surface pattern recognition receptors (PRRs) that stimulate complex immune responses (Ngou et al., 2022). PRRs are either receptor kinases or receptor-like kinases (RKs and RLKs) consisting of an extracellular receptor domain, a transmembrane domain and an intracellular protein kinase domain or receptor like proteins (RLPs) that lack the kinase domain (Couto & Zipfel, 2016).

Well-characterized PRRs from *Arabidopsis thaliana* are flagellin-sensitive 2 (FLS2) and the EF-Tu receptor that detect peptide motifs from, respectively, bacterial flagellin (flg22) and EF-Tu (elf18), (Couto & Zipfel, 2016; Miya et al., 2007). Chitin elicitor receptor kinase 1 (CERK1) recognizes in *A. thaliana* together with Lysin-motif proteins (LYM1 or LYM3) PGN and together with the LysM-containing receptor-like kinase 5 (LYK5) chitin (Willmann et al., 2011). In rice, CERK1 cooperates for PNG and chitin perception with, respectively, the lysin motif-containing proteins LYP4 and LYP6 and chitin elicitor binding protein (Hayafune et al., 2014; Liu et al., 2012). The detection of PAMPs by PRRs triggers complex signaling cascades in which protein kinases and the specific phosphorylation of signaling

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proteins play crucial roles (Bigeard et al., 2015; Zhang & Zhang, 2022). Early responses such as reactive oxygen production (ROS) and mtogen-activated protein kinase (MAPK) activation are triggered within minutes after PAMP treatment, whereas the activation of defense responses including, for example, cell wall reinforcement, transcriptional reprogramming and the production of secondary defense metabolites takes several hours (DeFalco & Zipfel, 2021; Katagiri & Tsuda, 2010).

A crucial link between PRRs and downstream signaling modules are members of the receptor-like cytoplasmic kinase (RLCKs) family. RLCKs belong to the large RLK superfamily, which is divided into 13 families (RLCK I–XIII) (Lehti-Shiu et al., 2009; Shiu et al., 2004). By contrast to RLKs, RLCKs lack the extracellular receptor domain and, in most cases, also the transmembrane domain. The RLCK VII subfamily, which can be further divided into nine subclades, is particularly important in plant immunity. Frequently, its members are phosphorylated by PRRs and thereby act immediately downstream of this important immune receptor class. Well-studied examples are BIK1 and PBL1, which are members of *A. thaliana* AtRLCK VII-8. They interact with FLS2 and the co-receptor RLK BAK1 upon detection of flg22 and are required for numerous responses such as a ROS production and callose deposition (Kadota et al., 2014; Zhang et al., 2010). In rice, OsRLCK176 and OsRLCK185 from the RLCK VII subclades 8 and 1 interact with CERK1 and positively regulate responses to peptidoglycan and chitin (Ao et al., 2014; Yamaguchi et al., 2013). OsRLCK185 is directly phosphorylated by OsCERK1 and *OsRLCK185* RNAi-lines lose chitin-induced MAPK activation and defense gene expression (Wang et al., 2017; Yamaguchi et al., 2013). In certain cases, the function of RLCK VII members in immune signaling appears to be conserved between monocots and dicots. Indeed, PBL27 from *A. thaliana*, such as OsRLCK185, its orthologue from rice, mediate immune responses triggered by chitin (Shinya et al., 2014).

Because of the important role of the RLCK VII family in plant immunity, pathogen effectors heavily target its members. For example, the bacterial effectors AvrPphB and AvrAC suppress PTI in dicot plants by targeting BIK1 and several other PBLs from the RLCK VII subfamily (Feng et al., 2012; Zhang et al., 2010). In rice, the *Xanthomonas oryzae* effector Xoo1488 targets OsRLCK185 to inhibit chitin-triggered immunity (Yamaguchi et al., 2013).

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has been developed as a powerful tool for genome editing in a broad range of organisms including bacteria, yeast, animals and plants (Xing et al., 2014). It exploits the extremely precise targeting of the Cas9 endonuclease from *Streptococcus pyogenes* to a 20-nucleotide DNA target sequence followed by an NGG protospacer adjacent motif (PAM) by a synthetic single-guide RNA (gRNA). The Cas9 nuclease creates double-strand DNA breaks approximately 3 nucleotides upstream of the PAM, which provokes deletion or addition of nucleotides as a result of errors during the repair by non-homologous enjoining (Jinek et al., 2012). CRISPR/Cas9-based multiple genome editing enables the creation of multiple DNA double-strand DNA breaks at specific sites in an organism's genome in a single mutagenesis step (Abdelrahman et al., 2021; Cong et al., 2013). It has been implemented for many plants, including rice, wheat, maize, tomato and *A. thaliana*, for the investigation of gene families where individual members have redundant roles and to improve crop traits, including disease resistance (Kumar et al., 2021; Li et al., 2013; Mao et al., 2019; Rothan et al., 2019; Tripathi et al., 2019). A particularly successful example is the editing of the sugar transporter genes *SWEET11*, *SWEET13* and *SWEET14* from rice to generate broad-spectrum resistance to the bacterial leaf blight pathogen *X. oryzae* pv *oryzae* (*Xoo*) (Oliva et al., 2019).

The RLCK VII-4 clade of *A. thaliana* was recently found to have a crucial and specific role in chitin-triggered signaling and immunity (Rao et al., 2018). We therefore wanted to analyze whether these functions of the RLCK VII-4 clade are conserved in monocot plants. For this, we inactivated, in rice, the six expressed *OsRLCK VII-4* genes by CRISPR/Cas9 multiplex genome editing and analyzed the resulting lines for their response to chitin and flg22 and for their immunity to *Xoo* and the rice blast fungus *Magnaporthe oryzae*. This revealed that the RLCK VII-4 clade of rice is required not only for chitin-triggered immune signaling, but also for the activation of flg22 responses. In addition, the RLCK VII-4 clade is necessary for the basal resistance of rice to bacterial and fungal pathogens.

RESULTS

Multiplex CRISPR/Cas9 allows inactivation of the OsRLCK VII-4 subfamily in rice

Using phylogenetic analysis and comparison with *A. thaliana*, we identified, among the 280 RLCKs of the rice reference variety Nipponbare, 54 genes that belong to the RLCK VII family (Lehti-Shiu et al., 2009). These 54 RLCKs could be further subdivided into eight clades, which match eight of the nine subfamilies in *A. thaliana* that we named accordingly (Figure 1) (Rao et al., 2018). Only the RLCK VII-3 subfamily has no members in rice.

In *A. thaliana*, members of the RLCK VII-4 subfamily (*PBL37*, *PBL38*, *PBL39*, *PBL40*, *PBL20* and *PBL19*) have crucial roles in immune signaling (Rao et al., 2018). However, because they have overlapping and redundant functions, phenotypes are only visible in higher-order mutants (Rao et al., 2018). To elucidate the role of the OsRLCK VII-4

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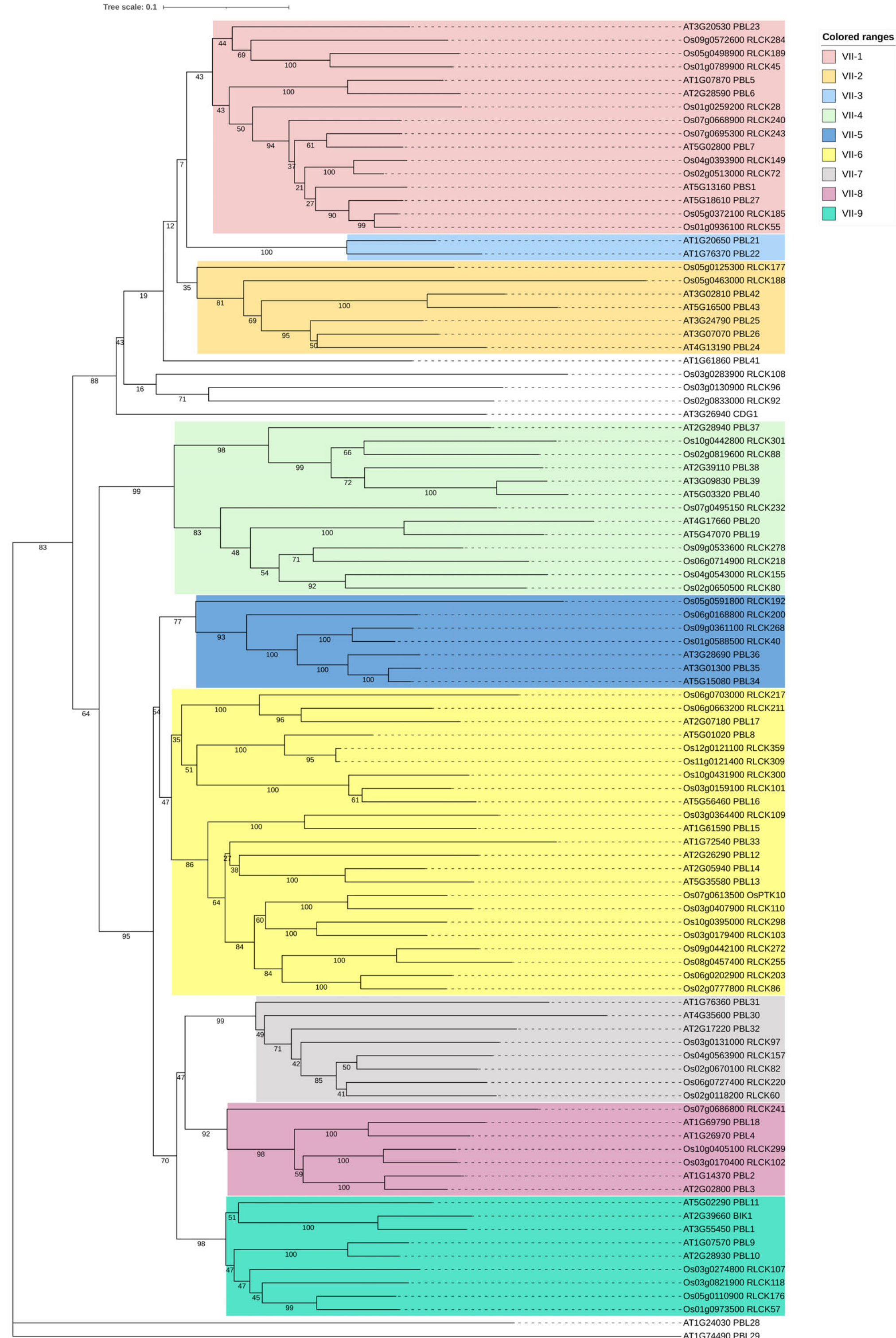

Figure 1. Comparative phylogenetic tree of the rice (*O. sativa*) and *A. thaliana* RLCK subfamily VII. The full-length protein sequences of RLCK VII members from rice and *A. thaliana* were aligned with MUSCLE. The alignment without gaps and missing data were analyzed with the maximum likelihood method with 1000 bootstraps to create a phylogenetic tree.

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subfamily in rice immunity, we therefore inactivated all OsRLCK VII-4 genes with significant expression in standard or stress conditions (Figure S1). Analysis of public gene expression data showed that only RLCK232 (Os07g0495150) was not expressed in leaves (Figure S1a) (Bidzinski et al., 2016). We therefore targeted the remaining six, *OsRLCK88*, *OsRLCK301*, *OsRLCK218*, *OsRLCK278*, *OsRLCK80* and *OsRLCK155* by multiplex CRISPR/Cas9-based genome editing (Xie et al., 2015). One gRNA was designed for each target gene and the resulting six sequences were cloned into a polycistronic tRNA-gRNA (PTG) gene (Figure S1b and Table S1). Transcripts from the PTG construct are cleaved by the endogenous tRNA-processing system to simultaneously produce the six individual gRNAs (Figure S1b).

The PTG/Cas9 construct was used for the creation of transgenic plants of the rice model variety Kitaake (Jain et al., 2019; Kim et al., 2013; Li, Jain, et al., 2017a). Among 126 independent antibiotic-resistant T_0 lines, 113 were confirmed by PCR to carry the *Cas9* gene. To identify mutations in the RLCK VII-4 genes, the gRNA target regions were amplified by PCR, and products were analyzed by cleavage with T7 Endonuclease1 (T7E1) that cuts heteroduplexes (Figure S2). This showed that all six RLCK VII-4 target genes were mutated at frequencies in the range 79–89% and that, in 65% of the transgenic lines, all six target sites were mutated simultaneously (Table 1). The eight sextuple mutant lines with the highest homozygosity rate were sequenced at the gRNA target sites, which confirmed all mutations with one single exception (OsRLCK218 in line 218) (Figures S2 and S3; Table S2). Single nucleotide insertions or deletions (InDels) were the most common type of mutation and occurred at a rate of 50%. Some 16% of the target sites carried two nucleotide InDels, 10% had three nucleotide InDels and 23% had higher order ones (Figure S1c and Table S2). The majority of the mutations were bi-allelic (27%), such that both copies of the target locus were mutated, and most were deletions (72%).

In four mutant lines (lines 189, 221, 263 and 306), all six target genes contained frameshift mutations leading to complete loss of function (Figure S2). The sequencing of putative off-target loci for the six gRNA that were identified with CRISPR-P showed that these four lines carried no additional off-target mutations (Figures S4 and S5; Table S3) (Lei et al., 2014). Therefore, for further analysis, we retained line 263, which had the highest rate of homozygosity because it was homozygous mutant for *RLCK 80*, *218* and *155*.

Screening of T1 generation plants of line 263 by PCR/T7E1 analysis and targeted sequencing identified three plants (263-7, 263-19 and 263-20) that carried homozygous frameshift mutations in all six targeted RLCKs (Figures S6 and S7). PCR analysis of the offspring from these plants identified multiple T2 individuals lacking the Cas9 transgene (263-7-1, -2 and – 4; 263-19-4 and – 6; and 263-20-2, -5, -7 and -11) (Figure S8). Seeds (T3) from these Cas9-free, homozygous sextuple OsRLCK VII-4 mutant plants and seeds from line 263-7 (T2) were used for phenotyping experiments and are subsequently designated as *rlck vii-4*.

The OsRLCK VII-4 subfamily regulates rice PTI

To investigate the role of the OsRLCK VII-4 subfamily in rice PTI, we first examined whether the ROS burst elicited by chitin- and flg22 was altered in the *rlck vii-4* multiplex mutant. A luminol-based chemiluminescence assay showed that ROS production triggered by both PAMPs was strongly reduced but not completely abolished in *rlck vii-4* plants (Figures 2a,b). In addition, we examined the activation of MAPK and callose deposition after chitin treatment. Analysis of MAPK-phosphorylation by western blotting showed that the activation of these important PTI signaling components by chitin was impaired (Figure 2c). The very rapid MAPK phosphorylation observed 1 min after chitin application was not altered, but the prolonged activation detected at 30 min was abolished in the *rlck vii-4* mutant (Figure 2c). Aniline blue staining of chitin-treated leaves revealed that chitin-triggered callose deposition was also drastically reduced in the *rlck vii-4* mutant compared to the wild-type (WT) (Figures 2d,e).

To further elucidate the role of the RLCK VII-4 subfamily in rice PTI, we analyzed the expression of the rice defense marker genes *PAL*, *PBZ1* and *PR10* after treatment with chitin and flg22 in *rlck vii-4* mutant and WT plants (Figures 2f,g). The induction of all three defense genes by

Table 1 Summary of the multiplex gene editing in OsRLCK subfamily VII-4

Target loci	Name	Guide sequence – PAM (5′- to 3′)	Cas9 positive	Mutant	Mutation rate %	Genotype[†]
OsRLCK88	gRNA1	TGAGTGCCGAATCAATACGGAGG	113	97	86	14 Ho+83 He+16 WT
OsRLCK301	gRNA2	GAGATCAGCGCCGAGTCGTTCGG		93	82	5 Ho+88 He+20 WT
OsRLCK218	gRNA3	CAGGAGCTGTCGGACGAGAGGGG		96	85	5 Ho+91 He+17 WT
OsRLCK278	gRNA4	CGTCAAGTGCCTGAACCAGCGGG		101	89	10 Ho+91 He+12 WT
OsRLCK80	gRNA5	GCGTCTACAAGGGCGTCGTTCGG		97	86	24 Ho+73 He+16 WT
OsRLCK155	gRNA6	GGACACGTTCGAGCACGCATCGG		89	79	10 Ho+79 He+24 WT

[†]Ho, He and WT are homozygote, heterozygote and wild-type, respectively.

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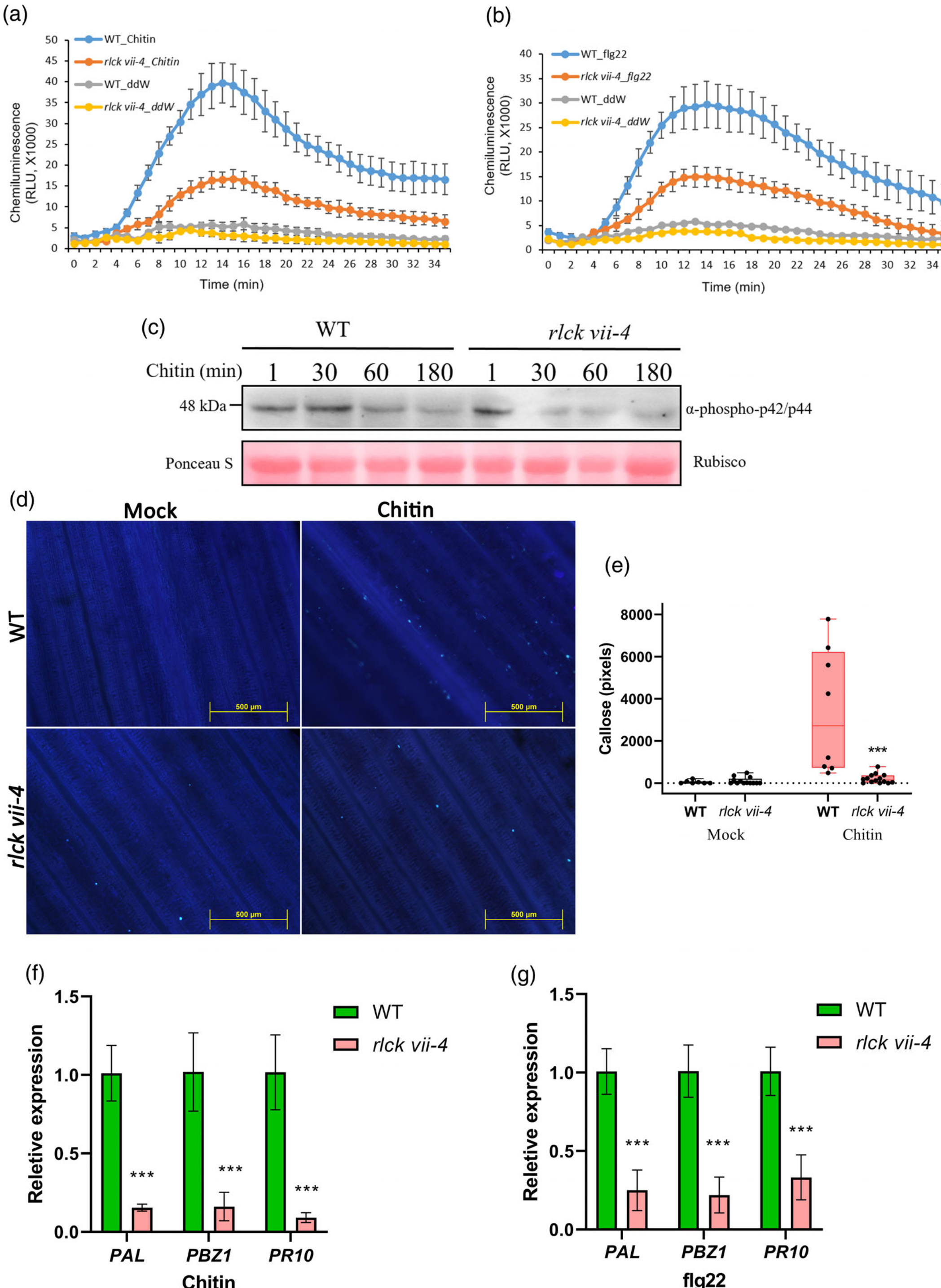

Figure 2. RLCK VII-4 members are required for PAMP-triggered ROS production, MAPK activation, callose deposition and pathogenesis-related gene expression. (a, b) ROS induction in *rlckvii-4* and wild-type (WT) plants. Leaf discs were treated with 1 mg ml^{-1} chitin (a) or 10 μM flg22 (b). ROS levels were determined by a luminol-based assay. The graph shows the mean ± SD calculated from three biological repeats. (c) Ten-day-old *rlck vii-4* mutant and WT seedlings were sprayed with 5 mg ml^{-1} chitin and harvested at the indicated time points. MAPK activation was analyzed by immunoblotting using a Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody. Equal loading is demonstrated by Ponceau S staining of Rubisco (bottom). (d, e) Leaves of *rlck vii-4* and WT were harvested 20 h after chitin or mock treatment and stained with aniline blue and observed. Images were captured with a fluorescence microscope under UV light (d) and were analyzed with GIMP (https://www.gimp.org) to quantify callose deposition (e). Bars represent the mean ± SD (n = 14 biological replicates and eight technical replicates). Statistically significance: ***$P < 0.001$, Student's t-test. (f, g) *PAL*, *PBZ1* and *PR10* expression in WT and *rlck vii-4* seedlings treated with chitin (1000 μg ml^{-1}) (f) or flg22 (10 μM) (g) for 3 h was analyzed by qRT-PCR using *Actin* as a reference gene. Data are the mean ± SD (n = 3 biological replicates and two technical replicates). Asterisks indicate statistically significance (Student's t-test): ***$P < 0.001$.

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both elicitors was drastically reduced in the mutant, indicating an important role of the RLCK VII-4 subfamily in the control of the transcriptional reprogramming accompanying the establishment of PTI.

Taken together, these analyses show that early PAMP signaling (i.e. ROS production and MAPK activation) and later defense responses (i.e. callose deposition and the induction of defense gene expression) are impaired in *rlck vii-4* mutant plants. This suggests that members of the rice RLCK VII-4 subfamily have important regulatory roles in the control of PTI signaling, such as in dicot plants.

The OsRLCK VII-4 subfamily is required for immunity to the fungal and bacterial pathogens *M. oryzae* and *Xoo*

To evaluate the role of the OsRLCK VII-4 subfamily in the immunity to bacterial and fungal pathogens, we compared the infection phenotypes of the *rlck vii-4* mutant and two transgenic control lines carrying a *monomeric red fluorescence protein* (*mRFP*) transgene after inoculation with the blast fungus *M. oryzae* and the bacterial leaf blight pathogen *Xoo*. The *mRFP* control line had been created in parallel to the *rlck vii-4* multiplex mutants. For the *rlck vii-4* mutant, we used three different T3 families originating from different Cas9-free T2 plants.

For *M. oryzae*, we used two different strains. The reference isolate Guy11, which causes disease on Kitaake and therefore allows an evaluation of changes in partial resistance to *M. oryzae*, and a transgenic strain of Guy11 that expresses the avirulence effector AVR-Pia, which is detected by the NLR pair RGA4/RGA5 in Kitaake. This strain therefore allows the assessment of effector-triggered immunity (ETI) (Okuyama et al., 2011; Yoshida et al., 2009). Instead of the WT allele of AVR-Pia, we used the R43G point mutant where the arginine at position 43 is replaced by glycine. This mutant of AVR-Pia induces a weaker resistance response, therefore allowing it to be more sensitive in the detection of an eventual attenuation of ETI (Ortiz et al., 2017). Inoculation with the incompatible Guy11-AVR-Pia_R43G isolate caused typical small HR lesions on all lines (Figure 3a). No significant difference was observed between the *rlck vii-4* mutant plants and the *mRFP* controls, such that the number and size of lesions were indistinguishable. This indicates that the OsRLCK VII-4 subfamily is not required for the immunity conferred by the NLR pair RGA4/RGA5. In the compatible interaction with the Guy11 WT isolate, typical disease lesions characterized by a gray center were formed (Figure 3a). *rlck vii-4* mutant plants developed much stronger symptoms than controls and had more and bigger lesions. This indicates that susceptibility to blast disease is increased in the *rlck vii-4* mutant, suggesting that the OsRLCK VII-4 subfamily is important for basal immunity to the fungus.

For bacterial infection assays, we used *Xoo*, which causes disease on Kitaake. Leaf clipping assays revealed more severe disease symptoms on the *rlck vii-4* line than on the controls, and the length of disease lesions and the lesion area was significantly increased by, respectively, 61.8 and 46% (Figures 3b,c). This shows that the OsRLCK VII-4 subfamily genes are involved in the control of basal resistance to *Xoo*. Taken together, infection experiments therefore indicate that the OsRLCK VII-4 subfamily acts in the basal immunity of rice to various pathogens.

DISCUSSION

Highly efficient multiplex target gene mutagenesis by CRISPR/Cas9 in rice

In the present study, using a PTG/Cas9 multiplex system, we successfully generated a sextuple mutant in rice variety Kitaake where the entire RLCK VII-4 subfamily was inactivated. The Kitaake variety was used because it is increasingly considered as a model for functional studies in rice and provides numerous advantages over other rice accessions such as a high regeneration rate, a very short life cycle (12 weeks), high yield, well studied resistance genes such as the NLR pair RGA4/RGA5, and high quality genomic resources (Jain et al., 2019; Kim et al., 2013; Li, Jain, et al., 2017a).

The individual RLCK VII-4 genes were mutated at an average frequency of 84.5%, and all six target genes were simultaneously mutated in 65% of the transgenic lines. This high mutation rate confirms the robustness of the PTG/Cas9 system in rice for which similar multiple genome editing efficiencies above 85% were reported in previous studies (Xie et al., 2015). A prerequisite for such elevated mutation efficiency is the careful design of gRNAs. Indeed, the GC content of gRNAs influences the multiplex CRISPR/Cas9 rate, probably by controlling the efficient binding of gRNAs to their target sites. We therefore took care to ensure that the GC content in all gRNA sequences was above 50% and not higher than 65% (Ma et al., 2015).

Similar to other CRISPR/Cas9 genome editing studies conducted in various plant species such as rice and *Arabidopsis*, deletion mutations were much more frequent than insertions (72 versus 28%), and single nucleotide mutations, which occurred at a rate of 50%, dominated over other, higher order-mutations (Endo et al., 2015; Feng et al., 2014; Hyun et al., 2015; Mikami et al., 2015; Zhang et al., 2014).

Screening for multigene knockouts was facilitated by the high frequency of bi-allelic mutations at multiple target sites. Furthermore, mutants could be self-pollinated to remove the T-DNA containing the PTG/Cas9 sequences and generate transgene-free mutants. This removes the possibility of positional effects from random T-DNA integration (Figure S7). As in other high-precision CRISPR/Cas9 studies in plants, we did not encounter off-targets in our sextuple mutant lines, which is a further asset of the chosen approach (Table S4) (Feng et al., 2018; Nguyen et al., 2021).

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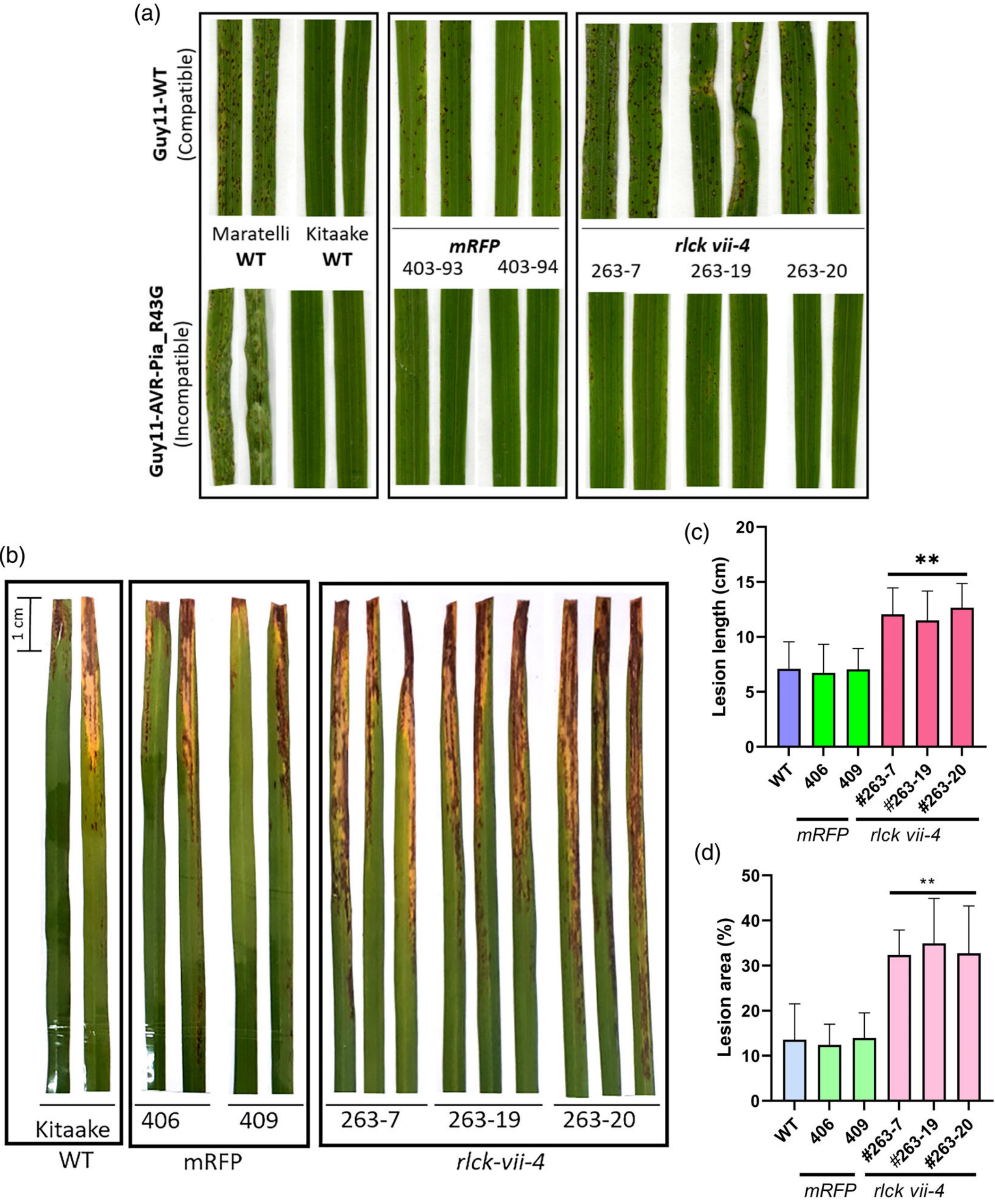

Figure 3. OsRLCKVII-4 positively regulates basal resistance against *Xoo* and *M. oryzae*. (a) Photographs of rice leaves taken 7 days after *M. oryzae* inoculation. Three-week-old rice plants of the *rlck vii-4* mutant (#263-7, #263-19 and #263-20), the *mRFP* control line (403–93 and 403–94), Kitaake wild-type and the susceptible rice variety Maratelli were inoculated with the *M. oryzae* strains, Guy11 carrying an empty vector or expressing the AVR-Pia allele R43G, which are, respectively, compatible and incompatible on Kitaake plants. Photographs show representative leafs 7 days after inoculation. (b) Photographs of rice leafs from *rlck vii-4* and *mRFP* plants 10 days after *Xoo* inoculation. (c) Lesion area and (d) lesion length on leafs of *rlck vii-4* and *mRFP* plants 10 days after inoculation with *Xoo*. Rice plants were inoculated with a bacterial suspension (OD_{600} = 0.6) by the leaf-clipping. Data presented (b, c) are the mean [WT (S = 11), 406 and 409 (S = 8), 263-7, -19 and -20 (S = 10)] ± SD and asterisks indicate a significant difference ($P < 0.05$, Student's *t*-test) compared to WT. The experiment was repeated twice with similar results.

RLCK VII-4 members regulate PTI in rice and basal resistance against bacterial and fungal pathogens

Different clades of the RLCK VII subfamily have important roles in immune signaling. The RLCK VII-8 clade that contains BIK1 and PBL1, which have critical roles in *A. thaliana* FLS2 and EF-Tu signaling and resistance to bacterial pathogens, has been particularly well studied. The orthologous clade in rice, which contains four members, OsRLCK 57, OsRLCK107 and OsRLCK118 and OsRLCK176, was reported to regulate chitin- and PGN-triggered immunity and

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immune responses controlled by the PRR Xa21 (Li, Ao, et al., 2017b; Zhou et al., 2016).

In Arabidopsis, the RLCK VII-4 subfamily composed of the six genes PBL19, PBL20, PBL37, PBL38, PBL39 and PBL40 was reported to be specifically involved in chitin-triggered immune signaling (Rao et al., 2018). A mutant line where all six AtRLCK VII-4 members were inactivated was defective for chitin-induced MAPK activation, ROS burst and defense gene expression, and showed enhanced susceptibility to the bacterial pathogen *Pseudomonas syringae*. However, this *A. thaliana rlck vii-4* sextuple mutant was not altered in its response to the PAMPs flg22 and elf18 (Rao et al., 2018). Mutant lines, where lower numbers of *AtRLCK VII-4* members were mutated, had weaker or no chitin response phenotypes indicating redundancy in this RLCK subfamily.

Previously, only one single rice RLCK VII-4 member, OsRLCK278 also named Broad-Spectrum Resistance 1 (BSR1), had been investigated. Mutant analysis showed that it is required in suspension-cultured cells for the full activation of a ROS burst and defense gene expression in response to the PAMPs chitin, PGN and lipopolysaccharide (Kanda et al., 2019). Overexpression of OsRLCK278 confers broad-spectrum resistance to fungal and bacterial pathogens and knockdown reduced basal resistance (Kanda et al., 2017; Maeda et al., 2016; Sugano et al., 2018).

In the present study, we found that complete loss of the RLCK VII-4 subfamily in the rice sextuple *rlck vii-4* mutant results in a drastic reduction of the ROS burst and the defense gene expression elicited by two different PAMPs, chitin and flg22. This indicates that members of the RLCK VII-4 subfamily act in rice in at least two different PAMP signaling pathways and putatively also downstream of other PRRs. This clearly distinguishes them from the *A. thaliana* RLCK VII-4 subfamily, which is only involved in chitin signaling controlled by LysM domain RLKS, revealing a remarkable difference in monocot and dicot immune signaling.

In addition, we found that the chitin-induced activation of MAPK was strongly reduced in the *rlck vii-4* mutant. MAPKs act in numerous PRR signaling cascades and MAPK cascades are frequently activated downstream of RLKs and RLCKs in development and immunity (Zhang & Zhang, 2022). The RLCK VII-4 PBL19 from *A. thaliana* phosphorylates, among other client proteins, the MAPK kinase kinase MAPKKK5 and the MAPK kinase MEKK1 downstream of CERK1-mediated chitin perception to activate, respectively, MAPK3/6 and MAPK4 (Bi et al., 2018). The reduced chitin-responsive activation of MAPKs in the rice *rlck vii-4* mutant therefore suggests similar roles of the OsRLCK VII-4 members in early PAMP-triggered immune signaling, and thereby guide future studies on this topic. Finally, the chitin-induced deposition of callose was attenuated in the rice *rlck vii-4* mutant indicating a critical role of OsRLCK VII-4 members in the deployment of the full PTI response.

Infection experiments with the fungus *M. oryzae* showed that full resistance conferred by the rice NLR pair RGA4/RGA5, which recognizes the *M. oryzae* effectors AVR-Pia and AVR1-CO39, was not affected in the *rlck vii-4* mutant (Cesari et al., 2013; Césari et al., 2014; Guo et al., 2018; Ortiz et al., 2017). However, the basal resistance to virulent isolates of *M. oryzae* and *Xoo* bacteria was attenuated in the *rlck vii-4* mutant. This suggests that the OsRLCK VII-4 members have no important function in NLR-triggered immunity, but have crucial roles in the activation of immune responses that limit the colonization by virulent pathogens and probably rely on the perception of multiple PAMPs and numerous PRR signaling pathways.

Although the present study revealed an important role of the rice RLCK VII-4 subfamily in PTI and basal resistance, the precise function of its individual members in the control of rice immunity remains to be established. Subsequent work therefore should focus on the role of single OsRLCK VII-4 members in rice immune signaling to decipher their specific and shared functions. For this aim, the sextuple mutant can be complemented with individual members of RLCKs subfamily VII-4 or their combinations. This will allow determination of which members act in which PRR pathways and where any redundancies exist. Using tagged versions of the kinases in the complementation analyses, it will also be possible to address the molecular events that occur upon activation, such as post-translational modifications or re-localizations, and it will enable the search for interacting proteins that are either regulators or substrates regulated by these kinases.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Rice plants (*Oryza sativa*, subsp. japonica cv Kitaake, and Maratelli) were grown in a greenhouse at a temperature of 28°C with a relative humidity of 60–75%. Rice seedlings for quantitative real-time PCR (qRT-PCR), callose deposition, MAPK and ROS assays were grown in half-MS medium containing 3% sucrose under a cycle comprising 16 h of light (26°C) and 8 h of darkness (24°C).

Design and construct of CRISPR/Cas9

We used CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2) to design gRNAs and to predict potential off-target sites (Lei et al., 2014). For multiplex genome editing in Kitaake, a plant transformation vector was constructed, which coded for PTG composed of six different tandemly arrayed tRNA-gRNA units, where each gRNA contained a target-specific spacer (Xie et al., 2015). Individual tRNA-gRNA units were created by PCR with oligos comprising the target specific spacer sequences and using the plasmid pGTR (Plasmid #63143; Addgene, Watertown, MA, USA) as a template for the tRNA-gRNA scaffold sequences (Tables S5 and S6). The PCR products were purified (Illustra GFX PCR DNA and Gel Band Purification Kit; GE Healthcare, Chicago, IL, USA). Individual parts were ligated together by Golden Gate (GG) assembly with the T7 DNA

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Ligase (New England Biolabs, Ipswich, MA, USA) and the *Bsa*I restriction enzyme using a thermal cycler with the program: 40 cycles at 37°C for 5 min, 20°C at 10 min, and a final incubation at 20°C for 1 h. The GG assembly products were amplified by PCR with Go Taq DNA polymerase (Promega, Madison, WI, USA), purified, digested with *Fok*I (New England Biolabs), purified and ligated into the *Bsa*I digested pRGEB32 vector (#63142; Addgene) using T4 DNA ligase (New England Biolabs). The ligation product was transformed into *Escherichia coli* Mach1, and plasmids purified from positive colonies were verified by Sanger sequencing and used for the *Agrobacterium*-mediated rice transformation (Table S1).

Generation of rice mutant lines and genotyping

An *Agrobacterium tumefaciens* strain EHA105 containing the PTG CRISPR/Cas9 construct was used for genome editing in Kitaake. Rice transformation was performed as described (Toki et al., 2006). Leaf samples were collected from each seedling and powdered by TissueLyser-II (Qiagen, Valencia CA, USA) to extract genomic DNA with the CTAB method. Initial screening for transformations (T_0) was performed via PCR of the *Cas9* gene with primers oAJ060 and oAJ061. To select T_0, T_1 and T_2 lines with possible sequence changes in the spacer RNA target sequences, the target regions were amplified with specific primers (Table S7) and PCR products were digested with the T7E1 enzyme. In the first step, the PCR products were directly digested with the T7E1 enzyme and double bands indicated heterozygosity, whereas individual bands indicated hemoduplex mutants or WT. In the second step, samples that yielded single bands in step 1 were combined individually with an equivalent amount of PCR product from a WT sample, subjected to one denaturation/renaturation cycle in a thermocycler and digested with the T7E1 enzyme. Double bands indicated mutant allele samples (formed heteroduplexes with WT), whereas single bands indicated WT samples (homoduplex WT DNA). Candidate multiplex RLCK VII-4 mutant lines identified by this procedure were further characterized by PCR amplification and Sanger sequencing of the target regions of all six target genes.

Measurement of ROS levels

ROS levels were determined with a luminol-based chemiluminescence method using leaf discs (5 mm in diameter) from 4-week-old rice plants. Before treatment, leaf discs were incubated overnight in 96-well plates with 100 μl of sterile water. The next day, sterile water was removed and leaf disks were immersed in a buffer containing 99 μL of luminol (Bio-Rad, Hercules, CA, USA), 1 μL of horseradish peroxidase (Sigma, St Louis, MO, USA) from 100× stock solution (1 mg ml^{-1}). After the leaf discs were treated with 10 μM flg22 (GeneScript, Piscataway, NJ, USA), 1000 μg ml^{-1} chitin (YSK, Shizuoka, Japan) or water control, ROS production was monitored by a chemiluminescence assay. Luminescence was measured at 60-sec intervals for 35 min using a multimode plate reader (Perkin Elmer, Waltham, MA, USA). For each treatment, the mean ± SD were calculated from three biological replicates.

Callose detection

The leaves of *rlckvii-4* mutant lines and WT (10-day-old) were sprayed with a 1000 μg ml^{-1} chitin solution containing 0.02% Tween or a 0.02% Tween mock solution. Leave samples were transferred into an ethanol:acetic acid solution (3:1) for 5 h, dehydrated in 70% ethanol and 50% ethanol for 2 h, respectively, and then kept overnight in water. After washing four times with water, leaves were treated with 10% NaOH until the tissue became transparent (after approximately 1 h). The leaves were rinsed with water and shaken for 4 h in the staining solution (150 mM K_2HPO_4, pH 9.5, 0.02% aniline blue) (Yang et al., 2019). The callose deposition on the rice leaves was then observed under a UV light using a BX51 fluorescence microscope (340–380 nm; Olympus, Tokyo, Japan).

MAPK activation

Ten-day-old rice plants were sprayed with a solution of chitin (5 mg ml^{-1}) containing 0.02% v/v Silwet L-77. The youngest fully expanded leaves from five individual rice seedlings (90 mg) were harvested 1, 30, 60 or 180 min after the treatment and ground in liquid nitrogen. The resulting fine powder was re-suspended in 900 μl of extraction buffer (10 mM Tris–HCl, 75 mM NaCl, 15 mM EDTA, 15 mM β-glycerophosphate, 1 mM NaF, 0.5 mM Na_3VO_4, 1 mM DTT, 0.1% v/v Tween-20, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 12 000 ***g*** for 20 min the total protein concentration of the extract was determined using a BCA Protein Quantification Kit (Parstous Co., Mashhad, Iran). Then, 40 μg of total protein per sample were separated on a 12% w/v SDS-PAGE gel in accordance with the manufacturer's instructions (Mini-PROTEAN Tetra Vertical Electrophoresis Cell, #1658006FC; Bio-Rad), After transfer of the proteins to a poly(vinylidene fluoride) membrane using a semi-dry electrophoretic transfer cell (#1703940; Bio-Rad), activated MAPKs were detected using an anti-phospho-p44/42 antibody (dilution 1:1000; catalog. no. 9101; Cell Signaling Technology, Danvers, MA, USA) and an anti-rabbit HRP-conjugated secondary antibody (dilution 1:5000; Sigma). Chemiluminescence detection (G:BOX iChemi XT system; Syngene, Bangalore, India) was performed using ECL SuperSignal West Femto (#34094; Thermo Fisher Scientific, Waltham, MA, USA).

RNA extraction and qRT-PCR analysis

Ten-day-old rice seedlings were sprayed with 1000 μg ml^{-1} chitin or 1 μμ flg22 (GeneScript) containing 0.025% v/v Silwet L-77 and, after 3 h, the youngest fully expanded leaves were harvested from five different plants for each biological replicate (three different biological replicates for each genotype and treatment). Total RNA was extracted with DENAzist Column RNA Isolation Kit (DENAzist Asia Co., Mashhad, Iran) and subjected to first-strand cDNA synthesis using an Easy cDNA Synthesis Kit (Parstous Co.). qRT-PCR was performed using a CFX96 Dx Real-Time PCR Detection Systems (Bio-Rad). The primer pairs oAJ103/oAJ104, oAJ105/oAJ106 and oAJ107/oAJ108 were used for *PAL*, *PBZ1* and *PR10*, respectively, whereas the oAJ109/oAJ110 primers were used for the rice *Actin* gene. The primers used for qRT-PCR are listed in Table S7. The transcript levels were calculated by the relative $2^{-\Delta\Delta Cq}$ (Livak) method with the rice *Actin* gene (Os03g50890) as the reference for normalization.

Pathology assays

For *Xoo*, 6–7-week-old rice plants were inoculated using the leaf clipping method (Ke et al., 2017). *Xoo* bacteria were cultured on solid PSA medium (10 g L^{-1} tryptone, 10 g L^{-1} sucrose and 1 g L^{-1} glutamic acid) at 28°C for 2 days and resuspended in 10 mM sterilized $MgCl_2$ solution at OD_{600} = 0.5. The tip of scissors was immersed in the *Xoo* suspension and approximately 2 cm was cut from the leaf tip. After inoculation, plants were kept at 28°C (16 h of light), 26 °C (8 h of dark) and 90% relative humidity. Lesion length and lesion areas were measured 12 days after inoculation. Two replicate experiments were performed where, each time, 15 plants per line were used for each treatment (one leaf per plant for each treatment).

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Magnaporthe oryzae isolates, Guy11 WT and Guy11-AVR-Pia_R43G (Ortiz et al., 2017) were grown for 10 days on rice flour agar. Spores was collected in water with 0.1% gelatin, adjusted to a concentration of 4×10^4 spores ml^{-1} and sprayed on 3-week-old rice plants (Berruyer et al., 2003). After inoculation, the plants were placed in a dark growth chamber at 26°C and 100% humidity for 16 h and then returned to normal growth conditions. Rice blast disease symptoms were analyzed 1 week after inoculation on the youngest leaf that was fully expanded at the time of inoculation.

Phylogenetic analysis

The amino acid sequence of all rice (cv Nipponbare) and *Arabidopsis* (Columbia) proteins was downloaded from the Phytozome v13 online database (https://phytozome.jgi.doe.gov) and the RLCK VII protein kinases were obtained using the iTAK website (http://itak.feilab.net/cgi-bin/itak/index.cgi). All RLCK VII protein sequences were aligned using MUSCLE (Edgar, 2004). Phylogenetic analysis was performed with MEGA7 (https://www.megasoftware.net/) using the maximum likelihood method with 1000 bootstrap replications. The iTOL online tool (https://itol.embl.de) was used to draw the phylogenetic tree with bootstrap values at the nodes.

Statistical analysis

All the data were analyzed using Student's *t*-test with Prism, version 9.0.0 (GraphPad Software Inc., San Diego, CA, USA), and this software was also used for graphical images. Values are shown as the mean ± SD.

ACCESSION NUMBERS

OsRLCK88 (Os02g0819600), *OsRLCK301* (Os10g0442800), *OsRLCK218* (Os06g0714900), *OsRLCK278* (Os09g0533600), *OsRLCK80* (Os02g0650500), *OsRLCK155* (Os04g0543000), *PAL* (Os02g0627100), *PBZ1* (Os12g0555500), *PR10* (Os03g0300400) and *Actin* (Os03g50890).

AUTHOR CONTRIBUTIONS

AJ, AB, TK and AMK designed the research. AJ performed the research. VC performed *M. oryzae* inoculation test. IM performed part of tissue culture. AJ, AB, TK and AMK analyzed the data. AJ, TK and AMK wrote the paper. All authors read and approved the final version of the manuscript submitted for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Multiplex genome editing of the rice RLCK VII-4 subfamily with a polycistronic tRNA-gRNA (PTG)/Cas9 system.

Figure S2. Identification of heterozygous mutations at six genomic sites in T_0 transgenic plants expressing PTG: Cas9.

Figure S3. Results of Sanger sequencing for multiplex genome editing in T_0.

Figure S4. Sanger sequencing of the putative off-target regions for multiplex mutant lines.

Figure S5. Analysis of the putative off-targets for multiplex mutant lines by PCR/T7E1 assay.

Figure S6. Identification of heterozygote and homozygote mutations in targeting sequences of gRNA2 for T_1 transgenic plants.

Figure S7. Multiplex genome editing for six target sites in T_1 line 263.

Figure S8. Segregation of Cas9 in line 263 in T_1 and T_2.

Table S1. Sequence of synthetic PTG gene used in the present study.

Table S2. Summary of genotyping and sequencing results for multiplex CRISPR/Cas9 mutant lines in T_0.

Table S3. Putative CRISPR/Cas9 off-target sites in the T_0 generation for PTG.

Table S4. Detection of mutations at the putative CRISPR/Cas9 off-target sites in the T_0 generation.

Table S5. Oligo nucleotides used to synthesize PTG gene.

Table S6. To construct PTG, the forward and reverse primers to amplify parts were added as indicated.

Table S7. Primers used for plasmid construction, QRT-PCR and genotyping.

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