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Research Paper

CRISPR/Cas9-Targeted Knockout of Rice Susceptibility Genes *OsDjA2* and *OsERF104* Reveals Alternative Sources of Resistance to *Pyricularia oryzae*

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Abstract: Rice blast, caused by *Pyricularia oryzae*, is one of the most destructive diseases in agriculture leading to severe impacts on rice crop harvests worldwide. In a previous study, we showed that rice genes *OsDjA2* and *OsERF104*, encoding a chaperone protein and an APETELA2/ethylene-responsive factor, respectively, were strongly induced in a compatible interaction with blast fungus, and also had their function in plant susceptibility validated through gene silencing. Here, we report the CRISPR/Cas9 knockout of OsDjA2 and *OsERF104* genes resulting in considerable improvement of blast resistance. A total of 15 *OsDjA2* (62.50%) and 17 *OsERF104* (70.83%) T_0 transformed lines were identified from 24 regenerated plants for each target and used in downstream experiments. Phenotyping of homozygous T_1 mutant lines revealed not only a significant decrease in the number of blast lesions but also a reduction in the percentage of diseased leaf area, compared with the wild-type infected control plants. Our results supported CRISPR/Cas9-mediated target mutation in rice susceptibility genes as a potential and alternative breeding strategy for building resistance to blast disease.

Key words: gene editing; plant-pathogen interaction; *Magnaporthe pathosystem*; plant immunity; blast resistance; *S*-gene; rice

Rice (*Oryza sativa* L.), the staple food for more than half of humankind, is a crucial crop for food security, feeding more people than any other cereal crop (Chen et al, 2018). However, rice plants have to deal with *Pyricularia oryzae* (synonym *Magnaporthe oryzae*), a hemibiotrophic fungus responsible for rice blast, one of the most ubiquitous and destructive diseases affecting rice production globally (Jain et al, 2017). The cultivation of rice resistant varieties, harboring single or a couple of major resistance (*R*) genes, is the most used and environment-friendly approach to cope with *P. oryzae* infection (Ahn and Seshu, 1991). Nonetheless, along with being a labor-intensive technique, conventional breeding aiming at *R* gene-mediated resistance is race-specific and partially efficient. Moreover, resistance is often broken down within a few years after its commercial use (Bonman et al, 1992).

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Alternatively to the resistance governed by *R* genes, the genetic manipulation of host susceptibility (*S*) genes represents a powerful source towards a more durable rice-blast resistance (Zaidi et al, 2018). Although the plant-pathogen arms race has forced pathogens to continuously evolve new strategies to evade or suppress plant immunity, most pathogens require host cooperation for the establishment of a compatible interaction, and typically exploit hosts' *S*-genes to facilitate their nutrition and proliferation (Win et al, 2012). Hence, all plant genes that somehow facilitate infection and/or support compatibility can be considered as an *S*-gene (van Schie and Takken, 2014).

Further investigations of different pathosystems assisted by omics (e.g., proteomics and transcriptomics) together with gene silencing technologies (e.g., Antisense Oligonucleotide - ASO, Host-Induced Gene Silence - HIGS and RNAi) have expanded our understanding of the molecular basis of pathogenicity, revealing crucial players (potential candidate *S*-genes) engaged with the infection process, and notably contributed to the ever-expanding host *S*-gene repertoire. More recently, CRISPR/Cas genome editing technology (Jinek et al, 2012) has offered new frontiers to overcome plant-pathogen compatibility by targeting *S*-genes in a very precise manner, enabling the development of transgene-free disease-resistant varieties, with several such cultivars already commercialized worldwide (Parisi et al, 2016).

In a previous shotgun proteomics study (Távora et al, 2021), we identified *OsDjA2* (LOC_Os02g56040) with a remarkably increased expression (3.58 [$\log_2$]-fold change) in a susceptible interaction at 12 h post-infection with *P. oryzae*. Aiming to reinforce the set of candidate target-genes, as well as to broaden the frame of prospection, a second potential candidate was picked from a transcriptomics study performed by Bevitori et al (2020). *OsERF104* (LOC_Os08g36920) was the most notable differentially expressed gene (DEG) (4.22 [$\log_2$]-fold change), identified 24 h post-infection (hpi) with *P. oryzae* in the same susceptible interaction. Further, we successfully characterized their function in rice susceptibility through an antisense gene silencing assay, where treated plants showed a notable decrease in foliar blast disease symptoms compared with control plants (Távora et al, 2021).

Here, the CRISPR/Cas9-target knockout (KO) of *OsDjA2* and *OsERF104* genes in the model *japonica* rice variety Nipponbare is reported. Homozygous mutant lines of T_1 progeny carrying edited forms of each targeted gene displayed enhanced resistance to blast disease. Therefore, although the molecular mechanism of rice susceptibility to *P. oryzae* is far from being fully captured, the precise and rational manipulation of host susceptibility genes can contribute to the development of effective disease management strategies, making it an interesting alternative and/or complementary approach to *R* genes in breeding programs.

RESULTS

ex-vivo assessment of sgRNAs gene-editing activity

To evaluate the efficacy of our CRISPR vectors in generating double-stranded break (DSB) at target sites, rice protoplasts were independently transformed with both constructs (*pCR_OsDjA2* and *pCR_OsERF104*) (Fig. 1-A and -B), and the results showed that our expressing vectors exhibited suited gene-editing activity on the target sites of rice protoplasts DNA (Fig. 1-C), hence, supporting their use for creating rice mutant plants.

Generation of *OsDjA2* and *OsERF104* rice mutant plants by CRISPR/Cas9 mutagenesis

We obtained 24 primary transformant (T_0) plants for each targeted gene. A total of 23 (95.83%) T_0 recovered plants of both *OsDjA2* and *OsERF104* were T-DNA PCR positive. The screening for T-DNA copy number integrated into their genomes by qPCR revealed 15 (62.5%) *OsDjA2* and 17 (70.83%) *OsERF104* primary transformant plants containing only 1–2 transgene copies, which were selected for further analysis. In those plants, CRISPR/Cas9-target mutagenesis was remarkably efficient: 93.3% and 70.6% of *OsDjA2* and *OsERF104* primary transformants, respectively, exhibited InDel mutations in the sgRNA target regions upon Sanger sequencing (Fig. 2-A). Regarding the nature of CRISPR/Cas9-induced mutations, further examination of sequence chromatograms revealed that among *OsDjA2* targeted alleles, there were 8 (57.1%) harboring biallelic mutations, 5 (35.7%) homozygous, and 1 (7.1%) heterozygous. Likewise, among *OsERF104* mutant lines there were 5 (41.6%) harboring biallelic mutations, 6 (50.0%) homozygous, and 1 (8.3%) heterozygous (Fig. 2-B).

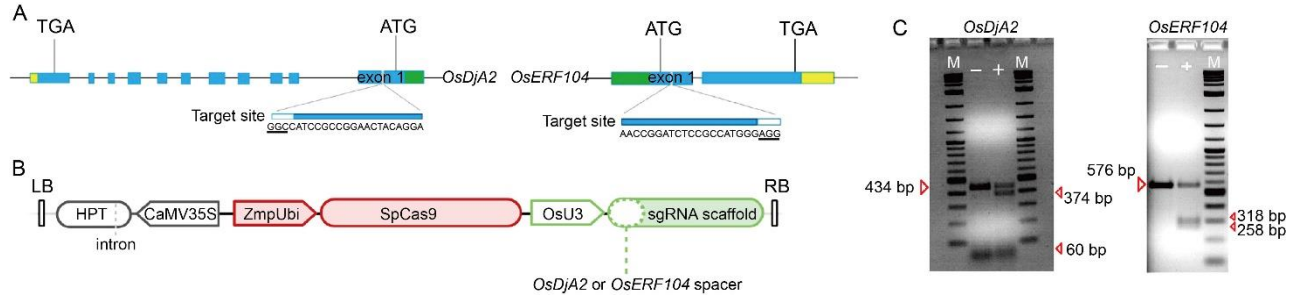

Fig. 1. CRISPR/Cas9 design and T7EI assay for sgRNAs gene-editing activity.

A, Schematic map of gRNA target sites on genomic regions of *OsDjA2* and *OsERF104*. Exons are indicated as blue boxes, interspaced by introns shown as lines; PAM motif is underlined and represented as white boxes.

B, Simplified schematic representation of CRISPR/Cas9 T-DNA structure.

C, Assessment of gRNAs cleavage activity of rice protoplast genomic DNA via T7EI assay. I and II represent the PCR products of *OsDja2* and *OsERF104* target sites, respectively. '–' means non-cleaved PCR product derived from 'wild-type' protoplast transformed with a control plasmid; '+' means cleaved PCR product derived from protoplasts transformed with CRISPR/Cas9 final vector.

Assessment of InDel impacts on both ORFs and targeted gene products

in-silico outcomes of ExPasy Translate tool revealed that all *OsDjA2* and *OsERF104* homozygous T_0 mutant lines exhibited a premature stop-codon very early on their ORFs (Fig. S1). The frameshift mutations observed on *OsERF104* T_0 homozygous mutant lines (i.e.: +1[A]bp and +1[T]bp) resulted in an extensive deletion of 185 amino acids (84% of the total protein content), vanishing with the whole transcriptional factor AP2/ERF domain, laying from 75 to 132 amino acid (Fig. S2-A). On the other hand, the InDel mutation on *OsDjA2* T_0 homozygous mutant lines (i.e.: -1[G]bp), despite generating great predicted deletion of amino acid content (77%), has conserved 90 amino acids of the native protein, comprising a great portion of the N-terminal conserved domain (known as 'J' domain), and the nascent part of the glycine-rich region ('G' domain) (Fig. S2-B). For this reason, we performed a complementary analysis to check the InDel impacts (at the biological activity level) on the targeted-gene cognate residual OsDjA2 protein. According to PROVEAN (Protein Variation Effect Analyzer) scores, generated based on query sequences of the two *OsDjA2* homozygous T_0 mutant lines, the large majority of observed amino acid deletions/substitutions were predicted as

A

Target gene	No. of total T_0 transgenic plants	No. of total T_0 transgenic plants harboring single copies of T-DNS	No. of total T_0 transgenic plants harboring site-mutations	Overall mutation efficiency
OsDjA2 (LOC_Os02g56040)	24	15	14	93.3%
OsERF104 (LOC_Os08g36920)	24	17	12	70.6%

B

Mutant genotype	No. of mutated T_0 plants (*OsDjA2*)
Homozygous	5
Biallelic	8
Heterozygours	1
Chimeric	0
Total	14

Mutant genotype	No. of mutated T_0 plants (*OsERF104*)
Homozygous	6
Biallelic	5
Heterozygours	1
Chimeric	0
Total	12

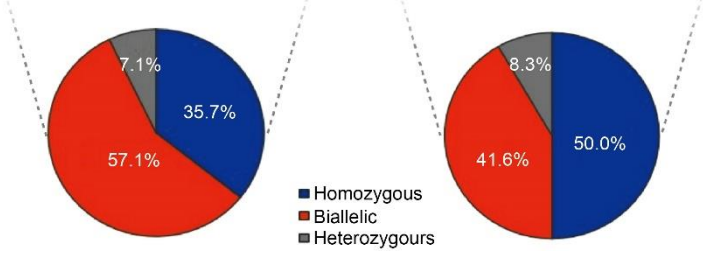

Fig. 2. Efficiency of CRISPR/Cas9-mediated genome editing of target genes *OsDjA2* and *OsERF104* (A), and ratios of mutant genotypes in mutated T_0 plants (B).

Overall mutation efficiency was calculated computing only the *OsDjA2* and *OsERF104* primary transformant plants containing only 1 or 2 transgene copy.

being deleterious (Fig. S3), which means that although OsDjA2 remained a truncated-protein, CRISPR/Cas9 mutagenesis probably led to the full knockout of the targeted-genes through loss-of-function (null) mutations.

Recovery of T_1 progeny homozygous mutant lines

A total of five independent T_0 mutant lines (*OsDjA2*_20.1 and *OsDjA2*_20.1_24.1; *OsERF104*_1.1, *OsERF104*_5.1 and *OsERF104*_6.1) harboring homozygous and predicted loss-of-function (null) mutations were selected and self-pollinated. T_1 progeny plants (n = 6 of each mutant line) were firstly screened for the presence of T-DNA (Fig. 3). All T_1 mutant events were homozygous for the same mutations observed in both *OsDjA2* and *OsERF104* T_0 parent lines (Table S1). It is noteworthy that no

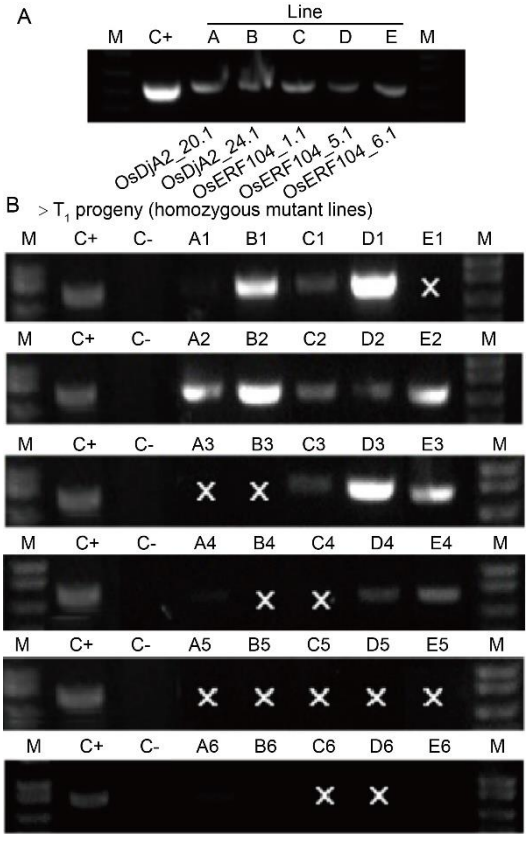

Fig. 3. PCR-based screening for the presence of T-DNA in rice mutant plants.

A, T_0 homozygous primary transformants (*OsDjA2*_20.1 and *OsDjA2*_24.1; *OsERF104*_1.1, *OsERF104*_5.1 and *OsERF104*_6.1).

B, T_1 progeny plants (n = 6 of each independent mutant line), using specific Cas9 primer pair (see Supplemental information). The letters (A–E) above the gel image refer to individual offspring (#1 to #6) of *OsDjA2* and *OsERF104* T_1 lines.

M, DNA molecular ladder; C+, CRISPR plasmid; C-, Genomic DNA of wild type Nipponbare; '×' indicates PCR negative for T-DNA.

unintended mutation was identified in the potential predicted off-target loci of our homozygous T_1 mutant lines (Table S2). Therefore, we were able to recover a sufficient number of suitable homozygous mutant rice plants to subject to the blast resistance assay.

Improved resistance to blast disease in CRISPR/Cas9-edited rice mutants

Wild type Nipponbare and T_1 homozygous mutant plants (*OsDjA2*_20.1 and *OsDjA2*_24.1; *OsERF104*_1.1, *OsERF104*_5.1 and *OsERF104*_6.1) of each targeted gene, with no detectable vegetative development defects under normal growth conditions (Fig. S4), were tested for blast disease resistance. All plants at the fourth-leaf stage were inoculated with the fungal pathogen *P. oryzae* compatible isolate GY0011. At 7 days post-inoculation (dpi), the number of blast lesions and the percentage of the diseased area on the fourth leaf of 20 plants (i.e.: independent biological replicates) of each mutant line were notably decreased in comparison with the WT inoculated control plants (Fig. 4-A). All tested replicates per mutant line of both targeted-genes were used for blast symptoms' quantification and are provided in the Fig. S5. The quantification of both disease severity parameters was further evaluated using a post-hoc Student's *t*-test which pointed to a statistical significance ($P < 0.05$) for the number of blast lesions on the leaves of *OsDjA2*_24.1 and *OsERF104*_5.1 mutant lines (Fig. 4-B), and likewise for the percentage of the foliar lesioned area on *OsDjA2*_24.1, *OsERF104*_1.1 and *OsERF104*_6.1 mutant lines (Fig. 4-C), in comparison with the WT inoculated control plants. Although some events from the same mutant line (*OsDjA2*_20.1 and *OsDjA2*_24.1; *OsERF104*_1.1 and *OsERF104*_5.1), harboring the same type of frameshift InDel mutations (-1[G]bp and +1[A]bp, respectively), showed a subtle deviation in disease severity phenotypes, such differences were not statistically significant ($P < 0.05$) when submitted to ANOVA test.

DISCUSSION

The plant pathogen *P. oryzae* poses a major threat to rice productivity worldwide. The fitness of susceptible rice cultivars is seriously impaired under disease pressure, leading to yield reduction or complete crop losses (Jain et al, 2017). To mitigate these negative impacts in agriculture, the usage of *R*-gene-containing cultivars has been for a long time the most effective measure for rice crop protection against blast disease

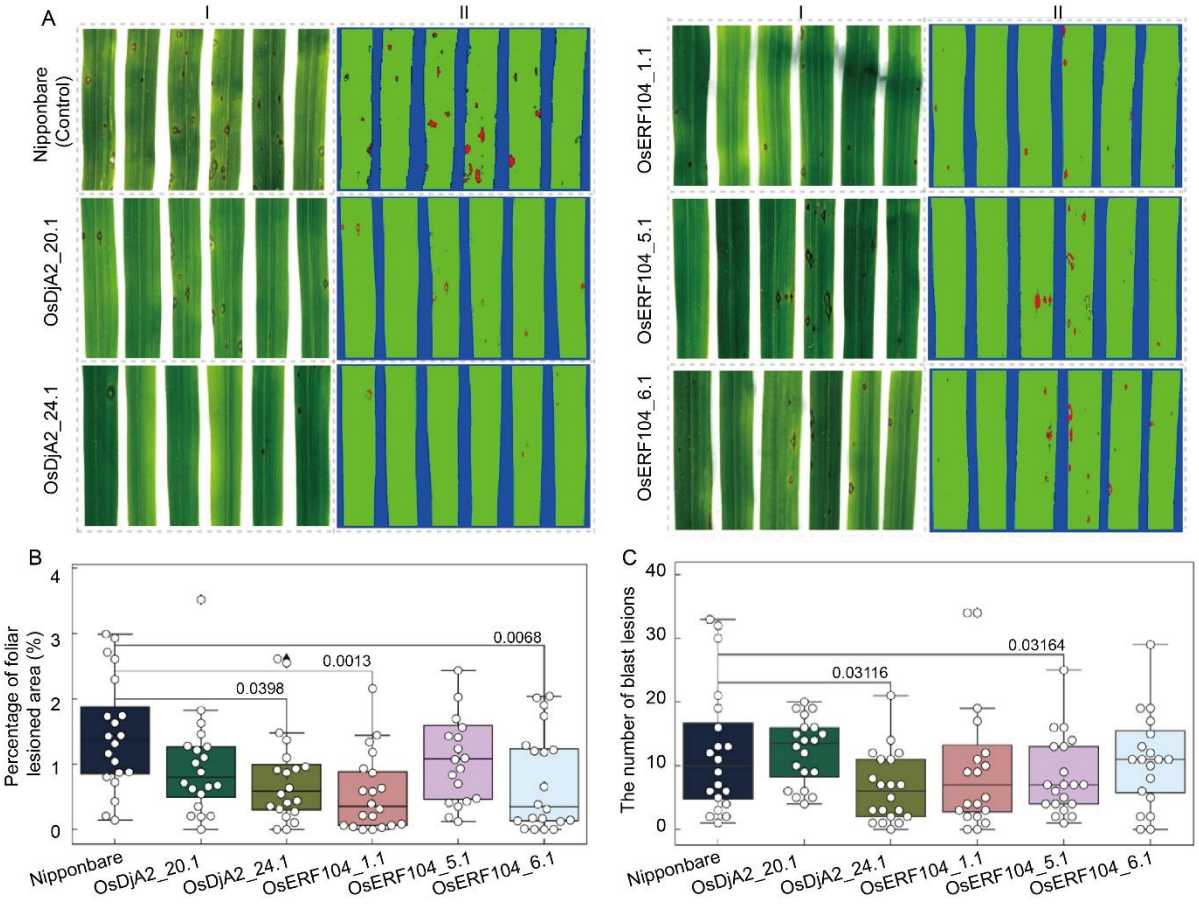

Fig. 4. Identification of blast resistance in CRISPR/Cas9-edited rice mutant plants.
A, Phenotypes upon blast infection of wild type Nipponbare (control), and T_1 homozygous mutant plants (*OsDjA2*_20.1 and *OsDjA2*_24.1; *OsERF104*_1.1, *OsERF104*_5.1 and *OsERF104*_6.1) of each targeted gene. The fourth leaves of 20 inoculated plants (independent biological replicates) per mutant and WT control line were detached at 7 days post-infection, scanned and analyzed for the number of blast lesions (A-I) and the percentage of the lesioned foliar area (A-II), using the software Quant®;
B and **C**, Boxplot merged with swarmplot data representation for the number of blast lesions (**B**) and the percentage of lesioned foliar area (**C**), respectively, observed on each of the 20 leaves of all mutant lines and control. The numbers above the boxplot indicate statistical significance ($P < 0.05$, two-sample *t*-test).

(Li et al, 2019). Nevertheless, dominant resistance governed by single *R* genes entangles several limitations (Stam and McDonald 2018). In this way, targeting host *S*-gene alleles re-flourished along with the recent advancements in new breeding techniques (NBTs), as an effective strategy to build a more durable and broad-spectrum disease resistance. Indeed, several case studies have been reported in this direction by mutagenesis of *S*-genes. A well-known and long-stablished host *S*-genes, *Mlo* (*mildew resistance locus O*), encodes a membrane-anchored protein that acts by supporting the establishment of fungus haustoria penetration structure facilitating the invasion of plant epidermal cells (Büschges et al, 1997). *Mlo* mutants represent the potential robustness of *S*-gene strategy, of which a recessive mutation was shown to confer powdery mildew (PM) resistance in barley seven decades ago and it continues to be employed and still confers durable resistance to all PM races in the field (Kusch and Panstruga, 2017).

Another great example of *S*-gene in rice is represented by the *SWEET* genes, with several members of this family already well characterized for their role in plant susceptibility to the bacteria *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Chen et al, 2010). This rice-pathogenic bacteria employs a set of transcription activator-like (TAL) effectors to function as specific transcription factors into plant cells, inducing the expression of plant *SWEET* genes (encoding putative sugar transporters), providing nutrients to the pathogen, thereby required for disease susceptibility (Streubel et al, 2013). More recently, Oliva et al (2019) used CRISPR/Cas9-mediated genome editing to introduce mutations,

simultaneously, in three sucrose transporter rice genes (*SWEET11*, *SWEET13* and *SWEET14*), whose expression are known to be required for disease susceptibility to *Xoo*, the causal agent of bacterial blight of rice. Authors demonstrated that CRISPR-edited SWEET promoters empower rice lines with robust, broad-spectrum resistance.

Other *S*-genes, encoding negative regulators of the first layers of plant defense responses (e.g., pathogen-triggered immunity-PTI or DAMP-triggered immunity-DTI), have also been reported. For example, phosphorylation-mediated MAP kinase (MAPK) signaling cascades are known to relay PTI activation, and these pathways are repressed by MAPK phosphatases (MKPs). Hong et al (2019) used CRISPR/Cas9 to reveal that rice *OsMAPK15* have similar PTI or salicylic acid (SA) defense suppressor activities. *OsMPK15* knock-out mutant significantly enhanced the disease resistance to different races of *M. oryzae* and *Xoo*, the causal agents of rice blast and blight disease, respectively.

In this context, we generated independent knockout mutant rice plants for *OsDjA2* and *OsERF104* putative rice-blast susceptibility genes that seem likewise to act in the first layers of plant defense. The present targeted genes *OsDjA2* and *OsERF104* were considered as potential host *S*-genes in our previous comparative proteomics investigation of rice-*P. oryzae* interaction coupled to a transient-inducible gene silencing assay (Távora et al, 2021). Their differential abundance (outstanding up to 4 [$\log_2$]-fold change increase) at the early stages of infection in the compatible interaction as well as the antisense oligo (ASO)-based gene silencing results, suggested their tight association with blast disease susceptibility.

From a total of 24 primary transformant recovered plants for each targeted gene, we achieved 15 and 17 single copy T-DNA *OsDjA2* and *OsERF104* events, respectively, of which 14/15 and 12/17 exhibited InDel mutations at their respective sgRNA target-sites (Table S3), implying a prominent efficiency of CRISPR/Cas9-target mutagenesis. We obtained 5 *OsDjA2* and 6 *OsERF104* homozygous T_0 mutant lines, harboring desirable frameshift InDel mutations (e.g., -1[G]bp, +1[A]bp, and +1[T]bp), which were self-pollinated and generated Cas9-positive plants as well as transgene-free T_1 progeny. The presence of the same InDel mutations at the targeted sites of T_0-derived plants and also the expected Mendelian segregation ratios of the transgene in the single copy mutant lines ultimately suggested stability of the inherited zygosity on the following generation. Therefore, we were able to recover a sufficient number of *OsDjA2* and *OsERF104* homozygous T_1 mutant lines showing on-target frameshift mutations on both alleles, the most suitable mutant rice plants for the phenotyping.

For the *OsDjA2* targeted-gene plants, 1/2 of the mutant lines showed significant ($P < 0.05$) improved disease-resistance (in terms of both analyzed parameters for disease symptoms (i.e.: number of blast lesions and percentage of foliar lesioned area). For *OsERF104* targeted-gene plants, 1/3 of mutant lines displayed a significant improvement in disease resistance in terms of the number of blast lesions, and 2/3 of mutants showed a significant decrease in the percentage of foliar lesioned area. Ultimately, inoculated mutant plants ($n = 20$ independent replicates per event) for both targeted-gene displayed a general trend of blast resistance in comparison with the wild type plants. Regarding the analyzed disease parameters in terms of *P. oryzae* pattern of infection, the observed results indicate an acquired blast resistance (partially, at least) probably due to an impairment of both fungus penetration and growth phases (inferred from the number of blast lesions and the percentage of foliar lesioned area parameters, respectively). Furthermore, especially in terms of the percentage of foliar lesioned area, the reduction of blast disease symptoms was significantly more pronounced ($P < 0.005$) on the *OsERF104* gene KO plants, suggesting a more critical role of this *S*-gene in host susceptibility, and probably reflecting the ubiquity of AP2/ERF transcription factor in plant stress responses and its broader engagement with rice-triggered susceptibility. Lastly, our results indicate that both *OsDjA2* and *OsERF104* rice *S*-genes seem to negatively regulate rice resistance to *P. oryzae* and their role and contribution to rice susceptibility is discussed below.

Exposed to an ever-changing environment, deluged by biotic and abiotic stressors, plants must be able to maintain cellular proteostasis for its proper growth, development, and survival (Park and Seo, 2015). This requires a fine-tune orchestration of a squad of molecular chaperones. Originally referred to as “Heat Shock Proteins” (Hsps) (Boston et al, 1996), these Hsps are indeed implicated in a myriad of functions in diverse plant species, playing also an essential and regulatory role in plant innate immune response.

Hsp70s and their obligate co-chaperones are known as J-domain proteins (JDPs), are arguably the most ubiquitous components of the cellular chaperone network (Verma et al, 2019). In addition, JDP (alternative names are currently used in the literature, such as DnaJ proteins, Hsp40 proteins, and J-proteins) represents the largest family of Hsp70 co-chaperones and are decisive for functionally specifying and directing Hsp70 functions. Rice genome counts for 115 J-protein family genes, randomly distributed on all twelve chromosomes, and classified into three classes (corresponding to types A–C) according to both domain organization and conserved signature sequences (Sarkar et al, 2013). Type A J-proteins, such as our *S*-gene target *OsDjA2*, are characterized by a 70 amino acid long J-domain, which is mostly present near the N-terminus, followed by a stretch of glycine/phenylalanine (G/F)-rich region, four repeats of a cysteine-rich CxxCxGxG-type zinc-finger motif, and a C-terminal domain involved in dimerization and substrate binding. In addition, the presence of a tripeptide motif His-Pro-Asp (HPD) is a highly conserved feature of the J-domain, argued to be essential for the stimulation of the ATPase activity of Hsp70s (Kampinga et al, 2019). Interestingly, our results of the *in-silico* prediction of InDel-induced frameshift mutations impacts on protein domains of *OsDjA2* T0 homozygous mutant lines showed that the protein residues lost their conserved HPD motif, as well as a great portion of the J-domain (showed in Fig. S2-C). The roles of HSP40/DnaJ proteins have been well studied in plant growth, development, and abiotic stress tolerance in plants. Regarding its function during biotic stress factors, we have pieces of evidence that in viral pathogenesis, for example, the silencing of diverse J-domain-containing protein can lead to resistance or susceptible outcomes (Luo et al, 2019; Ko et al, 2019). However, there are still large gaps in the understanding of how these DnaJ proteins negatively modulate plant immune response mechanisms during pathogen infection, in terms of PAMP sensing, signal transduction, and transcriptional activation/repression of stress-related genes, to trigger disease susceptibility, especially in crop plants.

Nonetheless, in the rice-*P. oryzae* pathosystem, consecutive studies have succeeded to functionally characterize the role of *DnaJ* homologue rice genes in blast susceptibility. The first study to link the expression of rice *DnaJ* gene in response to a fungal pathogen was made by Zhong et al (2018), where they reported the role of a chaperone DnaJ protein, *OsDjA6*, in the negative modulation of rice basal resistance upon *P. oryzae* infection. Researchers observed that the expression of *OsDjA6* was strongly induced early in a compatible interaction, and *OsDjA6* RNA interference (RNAi) mutated plants exhibited increased levels of reactive oxygen species (ROS) burst accumulation as well as up-regulation of defense-related genes, hence, enhanced resistance to *P. oryzae* in comparison with wild-type plants. More recently, Xu et al (2020) reported previously unidentified *P. oryzae*-infection tactics to trigger rice susceptibility. They found that MoCDIP4, an effector of *P. oryzae*, targets OsDjA9 protein to interfere with mitochondria-associated dynamin-related OsDRP1E protein complex, thereby inhibiting mitochondria-mediated plant immunity. Therefore, due to the great plasticity of DnaJ co-chaperone proteins, it seems that its novel role in rice susceptibility during *P. oryzae* infection starts to gather attention.

Another great player in the tangled modulation of plant immunity is plant hormones. Upon pathogen attack, ethylene (ET) phytohormone production typically raises and its complex signaling network can contribute positively or negatively to resistance depending on the enemy's lifestyle and tactics of infection (Anver and Tsuda, 2015). Phytohormone responses often are regulated by a large number of transcription factors (TF), with APETALA2/Ethylene Responsive Factor (AP2/ERF) family being the most conservatively widespread in the plant kingdom (Feng et al, 2020). According to Rashid (2012), there are 170 AP2/ERF plant-specific TF family genes in the rice (*Oryza sativa* L. spp japonica) genome and they are divided into a total of 11 groups, including the three most studied groups AP2, ERF, and DREB. The members of AP2/ERF gene family participate in different pathways in response to hormones and biotic/abiotic stresses, such a salicylic/jasmonate acid (SA/JA), abscisic acid (ABA), drought, salinity, cold, disease, and flooding stress (Phukan et al, 2017). Our CRISPR-edited rice gene *OsERF104* (generic name AP2/EREBP#152) is classified into the phylogenetic group IIIc of the rice ERF family (Nakano et al, 2006; Rashid et al, 2012), which is composed of 16 genes. The majority of its members have been found to integrate metabolic, hormonal and environmental signals in the biotic stress responses. *OsERF104*

encodes a plant-specific TF, containing only one APETALA2 (AP2) domain (of about 60 amino acids long) that plays decisive regulatory functions in controlling the transcription of downstream target genes by directly binding with cis-acting regulatory elements (called a GCC-box containing the core 5′-GCCGCC-3′ sequence) in their promoters. Interestingly, the *in-silico* computational prediction of the induced-mutation impacts on *OsERF104* gene product showed that the KO vanished with the whole transcriptional factor AP2/ERF domain (Fig. S2-A), argued to be vital for protein function in (a) biotic stress regulatory networks (Abiri et al, 2017). It is important to emphasize that the present targeted gene *OsERF104* was selected as a potential *S*-gene candidate from previous transcriptomics results, where it showed to be the most differentially-expressed gene (8.99 [log]-fold change; padj < 0.05) at 4 hpi in the susceptible interaction (Bevitori et al, 2020, raw data). In addition, it exhibited a notable differential increase at 12 hpi, and scored the highest fold-change 24 hpi in the susceptible interaction, compared with control plants (Bevitori et al, 2020). Although *OsERF104* also showed a differential increase in the resistant interaction, it is well known that pathogen-responsive genes are commonly expressed in compatible and incompatible interactions and are related to common defense pathways triggered by the pathogen (Ribot et al, 2008). The ERF genes are ubiquitous transcriptional factors, well-known for their plasticity and association with complex signaling networks, and roughly classified as activators or as repressors depending on whether they activate or suppress transcription of specific target genes (Srivastava and Kumar, 2019). Further, increasing evidence indicates that AP2/ERF TF may act as molecular switches to regulate clusters of stimuli responsive genes, playing key roles in both negative regulation of ET biosynthesis and rice susceptibility during distinctive plant-fungal interactions. For example, recent investigations have demonstrated different plant-specific ERF genes acting by suppressing PAMP-triggered immunity (PTI) upon pathogen infection. Lu et al (2020) revealed the importance of *AtERF019* gene (subgroup IIc) in mediating plant susceptibility to *Phytophthora parasitica* through the suppression of PTI and SA/JA defense responses. Likewise, it has also been reported that overexpression of *AtERF019* increases plant susceptibility to *Botrytis cinerea* and *Pseudomonas syringae*, and represses microbe-associated molecular patterns (MAMP)-induced PTI outputs (Huang et al, 2019). Regarding rice-*P. oryzae* pathosystem, in Liu et al (2012), authors observed a rapid and strong increase of ERF transcription factor *OsERF922* gene expression upon blast infection, and by means of RNAi gene silencing demonstrated that rice plant resistance phenotype was associated with a prompt increase in the expression of pathogenesis-related (PR) gene products. Later, Wang et al (2016) reported a great enhancement of rice resistance to *P. oryzae* through CRISPR knockout of the same *OsERF922* gene, asserting the role of this AP2/ERF domain-containing gene in rice-blast susceptibility.

Taken together, the appropriate manipulation of Type A J-domain and AP2/ERF TFs, suggested to be intricate with negative regulation of plant immune responses, has the potential to improve rice disease resistance. Our results revealed that CRISPR/Cas9-targeted KO of rice genes *OsDjA2* and *OsERF104* pointed to an enhanced resistance to *P. oryzae*, and also corroborates the findings of our previous work that suggested the ability of the blast fungus to modulate (early in the compatible interaction) the expression of a subset of rice *S*-genes, key players in the negative regulation of basal and innate plant-immune responses, favoring infection and host colonization. Lastly, the present study not only provides potential and alternative targets for fighting rice-blast disease but also strengthens CRISPR/Cas9-mediated knockout of rice susceptibility genes as a useful strategy for improving blast resistance.

METHODS

Plant materials and growth conditions

Rice cultivar Nipponbare (*Oryza sativa* L. spp. *japonica*) plants were grown in a Greenhouse facility at Cirad, France, under the following conditions: temperature of 28 ℃ during the day and 24 ℃ at night with 60% humidity. The natural light was complemented by artificial sodium light (700 μmol/[m ²s]). For blast inoculation, rice seeds from wild type Nipponbare (control) and T_1 progeny homozygous mutant lines were sown in rows (20 seeds per row) in 60 cm × 30 cm × 5 cm plastic seedling-nursing trays and maintained in greenhouse optimal conditions at the unit of Biologie et Génétique des Interactions Plante-Parasite – UMR BGPI, France.

Design of CRISPR/Cas9 sgRNAs and construction of T-DNA vectors

Gene-specific spacers (20 nt long sgRNA templates) for each

target *OsDjA2* and *OsERF104* (Fig. 1-A) were designed using CRISPR-assisted website (http://crispor.tefor.net/) (Concordet and Haeussler, 2018). We then inserted the sgRNAs into an entry vector derived from Miao et al (2013). Briefly, single-stranded gRNAs (20 nt oligos) were synthesized as spacer-containing primers (Table S4) and cloned into *BsaI*-digested pENTRY vectors. Subsequently, the resulting sgRNAs were cloned into the T-DNA region of a destination binary vector (Fig. 1-B), originally described by Miao et al (2013), in which minor modifications/improvements were made. The final CRISPR constructs for each target gene (*pCR_OsDjA2* and *pCR_OsERF104*) were confirmed by Sanger sequencing using specific primers (Table S4). Potential off-target mutations in CRISPR/Cas9-induced mutant plants were predicted by the CRISPOR tool (http://crispor.tefor.net/). For each of our target genes, we designed specific primers (Table S4) to amplify a genomic region (about 600 bp) flanking one top-ranking off-target site showing a higher likelihood to cause unintended mutations, and the resulting PCR products were analyzed by sequencing.

Rice protoplast for *ex-vivo* editing assay

Rice protoplast isolation and transformation were performed as previously described by Bes et al (2021). Briefly, Nipponbare seeds were sterilized in a 70% ethanol, 2.5% hypochlorite solution for 15 min under agitation, then washed five times in distilled water, and sown on 0.5× Murashige and Skoog (MS) solid medium (4.5 g/L phytagel) in rectangular (40 cm × 30 cm) Petri dishes. The seedlings were grown in the dark for 7 to 11 d at 26 ℃ in a growth chamber. Further protoplast isolation, purification, and transformation steps are detailed in the Supplemental data word file (Protocol).

Rice stable transformation

Rice transformations were carried out as previously described by Hiei et al (1994), with modifications. Briefly, *Agrobacterium tumefaciens* strain EHA105 was transformed, independently, with one of our previously described binary vectors (*pCR_OsDjA2* and *pCR_OsERF104)* by electroporation and then used for coculture with embryo-derived secondary calli tissue induced from mature seeds of wild type Nipponbare. We transformed a total of 30 calli per construction. Hygromycin-containing medium was used to select hygromycin-resistant calli that were then transferred onto regeneration medium for the regeneration of potentially transformed (edited) plants. After rooting and acclimation periods (approx. 3 months) into glass tubes, rice seedlings were transferred to soil in greenhouse optimal conditions.

Molecular characterization of CRISPR mutant events

We generated 24 hygromycin-resistant calli-derived regenerated plants for each CRISPR construction (*pCR_OsDjA2* and *pCR_OsERF104)*. Firstly, the genomic DNA of all primary transformants (T_0) was extracted by MATAB (Mixed Alkyl Trimethyl Ammonium Bromide) method and the presence of Cas9 in primary transformants and its segregation to the progeny was ascertained using the primers listed in Table S4. Transfer DNA (T-DNA) copy number was estimated by a DNA-based quantitative PCR (qPCR) optimized method (Yang et al, 2005) using *hpt*II-specific primers. The reaction and real-time fluorescence readings were carried out using a Light Cycler 480™ (Roche®). The copy number of the transgene was estimated after normalizing the amount of DNA using the reference gene and the DNA from a T_0 plant containing only one copy (verified by Southern blot) of the hygromycin gene as a comparison. The single-copy T-DNA sample served as a reference (for which it was assigned the value 1) and the transgene number of copies was estimated in relation to this reference value. The genetic material of T_0 plants harboring only one T-DNA copy were subjected to PCR using on-target specific primer pairs (Table S4) to amplify DNA fragments across both gene-target sites and amplicons subjected directly to Sanger sequencing. The generated chromatograms were explored and deconvoluted using CRISP-ID web-based tool (http://crispid.gbiomed.kuleuven.be/) (Dehairs et al, 2016) and CRISPR-mediated InDels on alleles of each mutant event were decoded. Lastly, we employed the ExPasy Translate tool (https://web.expasy.org/translate/) (Gasteiger et al, 2003) to provide a computational prediction of the impacts of CRISPR/Cas9-induced InDels on both the open reading frames (ORFs) of targeted-genes, and PROVEAN (Protein Variation Effect Analyzer) (Choi et al, 2012) algorithm, developed by Institute Craig Venter (http://provean.jcvi.org/index.php), to assess the variation effects caused by altered amino acid composition/chain structure on the biological function of its cognate-expressed proteins.

Pathogenicity assay

To evaluate the CRISPR-target KO mediated resistance to *P. oryzae*, the inoculation of rice blast fungus *P. oryzae* was performed as described by Sallaud et al (2003) Briefly, *P. oryzae* isolate GY0011, virulent (compatible) to Nipponbare, was cultured on oatmeal medium (20 g of oatmeal, 15 g of agar, 10 g of sucrose, and 1 L of distilled water) for 7 d in a dark incubator at 25 ℃. Conidia were harvested by flooding the plate with distilled water and softly scraping the medium surface. The concentration of conidial suspension inoculum was adjusted to 5×10^4 conidia/mL. Rice seeds of the control line and three independent homozygous T_1 mutant lines of each targeted gene were sown in trays of 20 cm × 12 cm × 5 cm filled with compost. Except for one independent homozygous mutant line (from the *OsDjA2* targeted gene) that did not germinate, all plants at the fourth-leaf stage were inoculated with *P. oryzae* by spraying with 20 mL conidial suspension per tray. The inoculated rice plants were stored for one night in a controlled dark chamber at 25 ℃ with 95% relative humidity and then transferred back to the greenhouse. Disease severity

was evaluated considering both blast lesion number per leaf and the percentage of lesioned leaf area, observed on the fourth leaves of 20 plants of each mutant and control line at 7 dpi using the software QUANT®, according to Vale et al (2003). Statistical analysis was performed using one-way ANOVA, followed by a post hoc two-sample *t*-test for average comparison between mutants and control line.

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SUPPLEMENTAL DATA

The following materials are available in the online version of this article at http://www.sciencedirect.com/journal/rice-science; http://ricescience.org.

Fig. S1. *in-silico* outcomes of ExPasy Translate tool.

Fig. S2. InDel-induced frameshift mutations on the protein domains of mutant lines.

Fig. S3. *PROVEAN* scores for the query sequences of mutant lines.

Fig. S4. Overview pictures of the recovered mutant rice plants of both targeted genes under greenhouse standard growth conditions.

Fig. S5. All 20 foliar replicates of wild type and T1 homozygous mutant rice lines of both targeted-genes tested for blast resistance.

Table S1. Segregation of CRISPR/Cas9-induced InDel mutations in sgRNA target regions of *OsDjA2* and *OsERF104* T_1 progeny that were submitted to the phenotyping.

Table S2. Analysis of off-target sites on mutant lines.

Table S3. CRISPR/Cas9-induced InDel mutations at both *OsDjA2* and *OsERF104* sgRNA cleavage sites on primary T_0 transformants.

Table S4. Primers used in this study.

Protocol. Rice protoplast for ex-vivo editing assay.

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