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Development of blast resistant rice plants using CRISPR / Cas9 system for genome editing

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Development of blast resistant rice plants using CRISPR / Cas9 system for genome editing

Thesis to the Post-Graduation Program in Biological Sciences required to obtain the Ph.D. degree in Biological Sciences, emphasis area Genetics and Biotechnology.

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Thesis´s defense presented to the Post-Graduation Program in Genetics and Biotechnology at the Federal University of Juiz de Fora, authored by Fabiano Touzdjian P. K Távora, entitled: **Development of blast resistant rice plants using the CRISPR / Cas9 system for genome editing**, as a requirement for obtaining the degree of Ph.D. in Genetics and Biotechnology, defined and approved by the undersigned examining jury:

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Dedication

I dedicate this study, fruit of four years of hard work, research, delivery and dedication, to my parents, foundations of my life, my lovely wife Taiana KTT Pinheiro, a person who knew to understand my absence due to all demands inherent of any scientific research and, finally, to my beloved and beautiful daughter, Dominique, sparkling source of inspiration, strength and hope.

To you, from the bottom of my heart.

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To EMBRAPA - Genetic Resources and Biotechnology, for all the support and collaboration;

To CAPES, for the academic and project financing.

EPIGRAPH

Once, already as a Ph.D. student, I was overcome by the noblest feelings of self-confidence and hope to move forward, when I heard a renowned researcher (a pity that his name escapes my memory at this very moment) open his speech as follows:

“(...) the researcher's career establishes in us deep and eternal marks of identity, such as the genuine love for knowledge, research and discovery, and the thrilling excitement of following the trail of an idea”.

RESUMO

Távora, Fabiano Touzdjian P. K. **Desenvolvimento de arroz resistente à brusone por meio do sistema *CRISPR/Cas9* para a edição de genomas**, 128 folhas. Tese de Doutorado em Genética e Biotecnologia – ICB/UFJF-MG, 2021.

O arroz (*Oryza sativa* L.) consiste na principal cultura alimentar de mais da metade da população mundial. Entretanto, esta cultura tem sido severamente atingida pela brusone, uma devastadora doença de plantas causada pelo fungo *Magnaporthe* oryzae. Dessa forma, o desenvolvimento de cultivares de arroz com maior resistência à brusone consiste em um dos principais focos dos programas de melhoramento. No entanto, devido à complexa biologia do patógeno, cultivares de arroz geneticamente resistentes ao fungo tornam-se suscetíveis em um curto período de tempo. O nocaute (deleção) de genes de suscetibilidade no genoma do arroz representa uma notável estratégia para a obtenção de uma resistência mais ampla e duradoura contra o fungo *M. oryzae*. O presente estudo teve como objetivo utilizar a tecnologia de edição genômica - sistema *CRISPR/Cas9*, para o nocaute de genes de arroz envolvidos na susceptibilidade à infecção fúngica. A partir de resultados anteriores de transcriptômica de duas linhagens semi-isogênicas de arroz - *NILs* submetidas a infecção por *M. oryzae*, foram selecionados potenciais genes de suscetibilidade. A prospecção por candidatos à edição gênica foi complementada por uma análise proteômica *shotgun* comparativa do perfil de proteínas da interação entre as *NILs IRBLi-F5* (suscetível) e *IRBL5-M* (resistente) em estágios iniciais da infecção por *M. oryzae*, que revelou um conjunto específico de proteínas potencialmente associadas à suscetibilidade. Após a caracterização e validação da expressão gênica por *RT*-qPCR dos candidatos mais proeminentes, os genes-alvos *OsDjA2*, *OsERF104* e *OsPyl5,* foram selecionados e submetidos a validação funcional via silenciamento gênico *in planta*, utilizando oligonucleotídeos antissenso (*ASO*), onde se observou notável redução dos sintomas foliares da doença na interação compatível. Em seguida, a variedade-modelo de arroz cv. *Nipponbare* foi transformada com os vetores *CRISPR/Cas9* visando o nocaute, independente, de cada um dos genes-alvo. Plantas de arroz da geração T1 e homozigotas para mutação-nula (perda de função) foram testadas quanto a resistência ao fungo *M. oryzae*. Conforme esperado, plantas editadas mostraram considerável redução dos sintomas da doença em relação às linhagens controle. Espera-se que os resultados obtidos contribuam para a geração de cultivares de arroz resistentes à brusone, além de lançar luz sobre novos potenciais genes de susceptibilidade à *M. oryzae*.

Palavras-chave: *CRISPR; Gene de susceptibilidade; Interação planta-fungo; Proteômica shotgun; Silenciamento gênico*

ABSTRACT

Távora, Fabiano Touzdjian P. K. **Development of blast-resistant rice through *CRISPR/Cas9* genome editing technology.** 128 pages. Thesis in Genetics and Biotechnology – ICB/UFJF-MG, 2021.

Rice (*Oryza sativa* L.) is the main food crop for more than half of the world population but unfortunately, it is severely affected by blast, one of the most widespread and devastating plant diseases, caused by the fungus *Magnaporthe oryzae*. Hence, the development of rice cultivars with greater resistance to blast is one of the main focuses of breeding programs. However, due to the complex biology of the pathogen, rice cultivars genetically resistant to the fungus become susceptible in a short period of time. In this context, the knockout of rice susceptibility genes represents a flourishing approach to obtain rice cultivars with a broader and longer-lasting resistance to *M. oryzae*. The present study aimed to use the genomic editing technology - CRISPR/Cas9 system, for knocking-out genes engaged with rice susceptibility to fungal infection. From previous transcriptomics results of two semi-isogenic rice lines - NILs infected by *M. oryzae*, potential rice-blast susceptibility genes were selected. The prospection of candidate genes for gene editing was complemented by a comparative shotgun proteomic analysis of the protein profile of the interaction between IRBLi-F5 (susceptible) and IRBL5-M (resistant) NILs in early stages of *M. oryzae* infection, that revealed a specific set of proteins potentially associated with susceptibility. After the characterization and validation of gene expression by RT-qPCR of the most prominent candidates, the target genes *OsDjA2*, *OsERF104* and *OsPyl5* were selected and submitted to a functional validation via gene silencing *in planta*, using antisense oligonucleotides (ASO), in which a clear reduction of leaf symptoms was observed in the compatible identification. Subsequently, the model japonica rice variety Nipponbare was transformed with simplex CRISPR/Cas9 vectors aiming to the independent knockout of each target gene. The T1 progeny of rice-edited plants, homozygous for the null (loss of function)-mutation were tested for blast resistance. As expected, mutant plants showed a decrease of disease symptoms in comparison with control lines (transformant non-edited plants). The results obtained in this study can contribute for the development of rice cultivars resistant to blast disease, besides shedding light on new potential rice-blast susceptibility genes.

Keywords: *CRISPR; Gene silencing; Plant-fungus interaction; Proteomic shotgun; Susceptibility gene.*

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Abbreviations

ANOVA	*Analysis of variance*
ASO	*Antisense oligonucleotides technology*
BRM	Brasil Microrganismo
BSA	*Bovine serum albumin*
CDS	*Coding sequence of a gene*
CID	collisional-induced dissociation
Cq	*Quantification cycle*
CRISPR/Cas9	*Clustered regularly interspaced short palindromic repeats*/Crispr-associated protein 9
DAP	*Differentially-abundant protein*
DDA	*Data-dependent acquisition*
Dpi	*Day post-inoculation*
DSB	*Double strand breaks*
dsRNA	*double-stranded RNA*
DTT	*Dithiothreitol*
EDTA	*Ethylenediaminetetraacetic acid*
ERF	*Ethylene-responsive factor*
ESI	*Electron spray ionization*
ETI	*Effector-triggered immunity*
ETS	*Effector-triggered susceptibility*
FDA	*Fluorescein diacetate assay*
FDR	*False discovery rate*
gRNA	*guide-RNA*
GMO	*Genetically-modified organism*
Hai	*Hour after infection*
HDR	*Homology-directed repair*
HR	*Hypersensitive reaction*
INDEL	*Insertion–deletion mutations*
IVT	*In vitro transcription*
LC-MS/MS	*liquid chromatography-tandem mass spectrometry*
LRR	*leucine-rich repeat*
LTH	*Lijiangxin-tuan-heigu rice cultivar*
MES	*2-(N-morpholino) ethanesulfonic acid buffer*
NEHJ	*Non-homologous end joining*
NIL	*Near isogenic line*
Pam	*Protospacer-adjacent motif*
PAMPs	*Pathogen-associated molecular patterns*
PCA	*Principal component analyses*
PCD	*Programmed cell death*
Pi5	*IRRI-blast resistance* gene
Pii	*IRRI-blast resistance* gene
PRR	*Pattern recognition receptors*
PS	*Phosphorothioate chemical modification type*
PSM	*peptide-spectrum matches*
PTI	*Pathogen-triggered immunity*
PTO	*Phosphorothioate antisense oligonucleotide*
RNP	*Ribonucleoprotein complex*
ROS	*Reactive oxygen species*
qPCR	*Quantitative real time PCR*
T7EI	*T7 endonuclease I enzyme*
TF	*Transcriptional factor*
TFA	*Trifluoroacetic acid*
XIC	*Extracted ion chromatogram*

General Introduction

Agribusiness and the relevance of Brazilian riziculture

Brazilian agribusiness is undoubtedly one of the main sectors that moves our domestic economy, significantly contributing to the national Gross Domestic Product (GDP). In 2018, the GDP of agribusiness increased by 1.87%, representing 21% of Brazilian GDP, which shows great power over the positive balance in the Brazilian trade balance (Center for Advanced Studies in Applied Economics - CEPEA). However, according to a FAO report, in 2050 the world population will be approximately 10 billion people, 29% more than the current number (available at: http://www.fao.org/brasil/noticias/detail-events /en/c/ 901168). In this context, there is a clear need to increase the world´s food production to meet this population growth forecast.

Rice represents the staple food of more than half of the world's population, contributing 23% of the total calories consumed globally. In addition, more than 600 million tons of this cereal are produced annually from 150 million hectares of rice paddies worldwide [1]. Brazil is the largest producer of upland rice, with a cultivated area of 2.3 million hectares, corresponding to 41% of Brazilian rice production [2]. Currently, Brazil occupies the 9th position in the World ranking of rice producing countries, with 11.9 million tons, standing out as the largest producer outside Asian continent [3], [4]. However, rice demands continue to increase as a result of increasing population and improving living standards, particularly in Latin America and African countries. It is estimated that we will have to produce ~ 30% more rice by 2030 [5]. Therefore, the application of innovative biotechnological products in agribusiness field is vital to respond to such global food demand, ensuring future sustainable production.

Food security in the current global scenario

In the current (and not optimistic) global scenario, in which adverse climate changes are associated with substantial population growth estimated at ten billion people by the end of 2050, food security represents one of the greatest challenges to be faced worldwide. Rice is the most important crop related to food security. However, due to its inherent high susceptibility to blast disease caused by the fungus *Magnaporthe oryzae*, associated to the difficulties in the disease management, rice yield is severely impacted, which leads to an annual striking of up to 10-30% of global production [6].

Scientific issues and working hypotheses

The setback of building resistance via conventional plant breeding

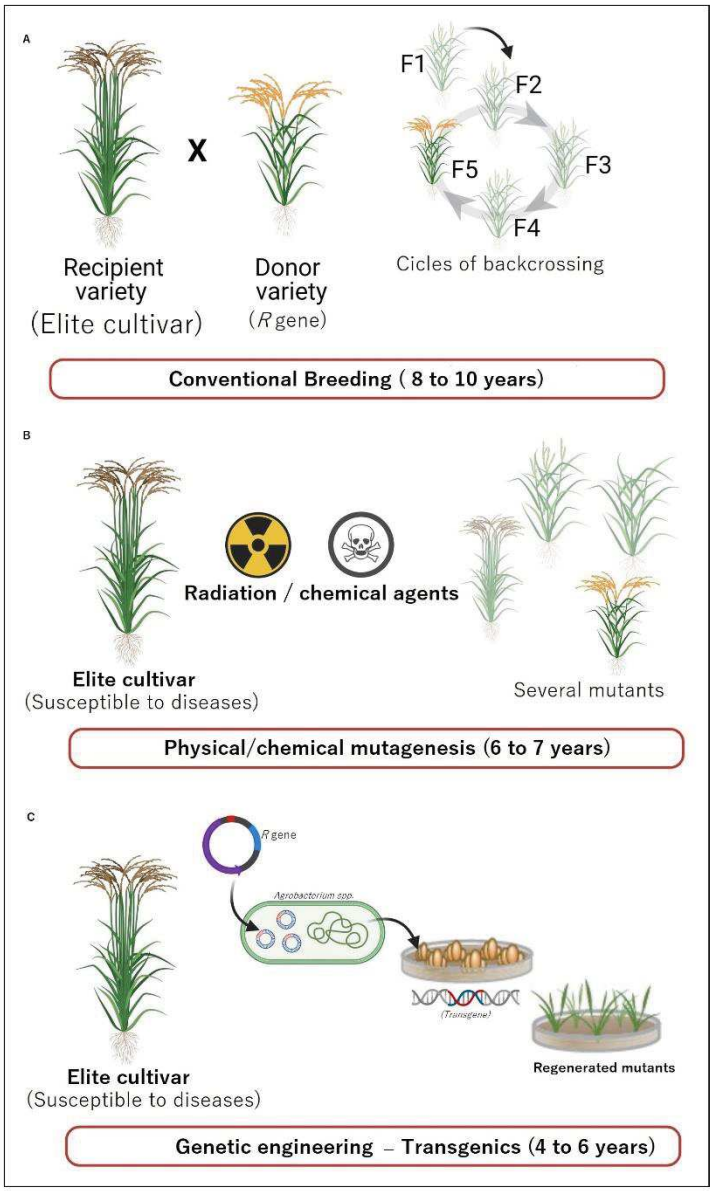

Figure 1. Comparative illustration of the main traditional breeding methods employed in the genetic improvement of rice (*Oryza sativa* L.) crop, aiming to generate disease-resistant varieties *(adaptation from Ahmad et al., 2020.)* (A) conventional plant breeding method, where an elite cultivar (recipient) presenting a high yield, but susceptible to diseases, is crossed with a resistant variety (donor of R-genes), generating a disease resistant plant. However, even after successive backcrosses and rigorous selection cycles, unwanted donor genes will be also incorporated (via linkage drag phenomenon) along with the desired ones; (B) genetic improvement via mutagenesis uses physical and chemicals agents (e.g., gamma radiation and ethyl methane sulphonate - EMS, respectively) to generate mutations in the plant's genome. In this process mutants must undergo rigorous selection during the evaluation of desirable phenotypes and one of the main limitations and disadvantages of this technique is the randomness of mutations in the genome and its tricky detection; (C) In plant breeding via genetic engineering (transgenics), in general, a gene of interest is isolated from a donor variety, cloned into a delivery vector and inserted into the genome of an elite host plant. The regenerated mutant plants will be regulated as a genetically modified organism (GMO) due to the insertion of exogenous DNA.

The development of rice cultivars with improved blast-resistance is one of the main focuses of conventional breeding programs. The cultivation of resistant varieties, containing one or more resistance R-genes, is one of the most environmental-friendly approaches to deal with *M. oryzae* infection. However, although breeders have managed to isolate several sources of genetic resistance, the high complexity of the pathogen and its ability to evolve into new races leads to a break in resistance in a short period of time [7]. In addition, the traditional techniques employed to generate these new varieties, such as conventional breeding, transgenics, chemical / physical mutagenesis (**Figure 1**) are, as a rule, laborious and require years to introduce desirable alleles and increase variability by genetic recombination [8].

New breeding techniques (NBTs): new horizon of resistances

Recent advances in the field of biotechnology have led to the emergence of the so-called 'new breeding techniques' (NBTs), whose performance has revealed alternative sources of plant resistance to the breeding programs, making susceptibility genes (S-genes) new targets for building a more durable and broad-spectrum pathogen resistance [9]. Among the NBTs, CRISPR / Cas9 system emerges as one of the most powerful and promising tools for a faster, more effective and sustainable genetic improvement of important agribusiness crops, such as rice (**Figure 2**).

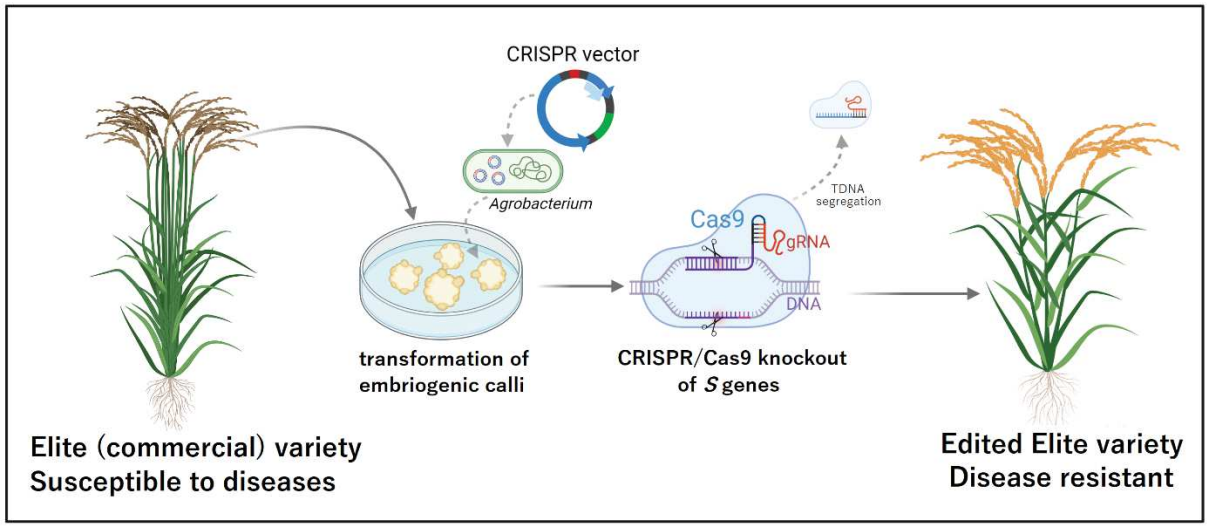

Figure 2. Illustration of rice breeding via the NBT, CRISPR / Cas9 system. In plant breeding via CRISPR/Cas system aiming, for example, to build pathogen-resistant varieties, a strategy that has been widely adopted is the deletion (knockout) of host susceptibility genes. Plant genetic improvement via genome editing is more accurate and efficient when compared to other traditional techniques. In addition, due to the absence of exogenous DNA (in the process and / or in the final product), the generated plant varieties tend, in several countries (according to the biosafety legislation), not to be considered as GMOs.

Literature overview

1. Rice: model plant for monocot studies

Rice (*Oryza sativa* L.) stands as the plant model system for monocotyledonous studies, mainly due to the publication of complete genome sequencing of spp. japonica cv. Nipponbare e spp. indica cv. 9311[10, 11], whose results placed rice into the spotlight of biological research (International Rice Genome Sequencing Project, 2005). After a quick search in GenBank (https://www.ncbi.nlm.nih.gov/nuccore/) using "*Oryza sativa*" as a species keyword, 2,390,616 items were retrieved. Another search on PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) using "rice" and "gene" as keywords, resulted in 20,558 articles, with 3,749 articles published from 1990 to 2004 (18.23%) and 16,610 articles published after 2005 (80.79%) (NCBI, 07/01/2019). Although inaccurate, the data reasonably reflects the rate at which rice researches were conducted after its whole genome sequencing. Currently, it is difficult to statistically determine the number of rice genes explored in basic research, however, predictions estimate around 1,000, amount considerably lower than the up to date sum of 37,544 genes present in rice genome[12]. Otherwise, it is easy to precise that these studies involved rice agronomic characteristics such as growth and development, as well as several aspects of rice-environment interaction (biotic and abiotic factors), so that all results contribute to a clearer and broader rice understanding. If genome sequencing has enabled a step forward in the increase and quality of rice research, subsequent technologies should then promote new directions in the field of rice bioengineering. The use of CRISPR/Cas9 system for genome editing, for example, has already shown its great potential in basic and applied rice research, surely a milestone of new possibilities in the field of rice functional genomics.

Among the benefits of using rice as a model plant, its genome size stands out with around 382 Mpb distributed in 12 chromosomes, the smallest among economically important cereals; the high density of genes (one gene every 8 Kb, approximately); the availability of high-density genetic maps, microarrays, and well-established genetic transformation methods, besides the vast germplasm of cultivated plants and wild species[12].

From the botanical perspective, rice plant is a *Liliopsida* (Monocot) from the *Poacea* (Gramine) family, subfamily *Pooideae*, tribe Oryzae (**Figure 3a**). The genus *Oryza* comprises 23 species, 21 of which are wild (tetraploid, 4n = 48) and only 2 cultivated (diploid, 2n = 24): *O. sativa* and *O. glaberrima*, native from Asia and West Africa, respectively. The species is subdivided into 2 subspecies: indica, cultivated in a submerged system - which can vary from 5 to 20 cm of water lay (aquatic culture that comprises about 88% of the rice cultivated surface area in tropical zones); and japonica, used both in aquatic culture (in temperate zones) and in

upland culture (similarly to other cereals, such as wheat, rye, etc.) in the tropical zones (**Figure 3b**).

Figure 3. Morphological and cultivation characteristics of rice (*Oryza sativa* L.). (A) schematic representation of the main plant structures, zooming in the panicles (inflorescence or spikelet enclosing the seeds); (B) Representative image of upland (above) and aquatic (below) rice systems. *(Diagrams and images available at http://iac.sp.gov.br, with adaptations).*

2. The fungus *Pyricularia oryzae* and blast disease in rice

Posing one of the most serious obstacles to the expansion of rice cultivated global paddies, the hemibiotrophic fungus *Pyricularia oryzae* is the causal agent of blast disease of rice crop, one of the most destructive and severe fungal diseases [13]. Presenting a worldwide distribution, the *Magnaporthaceae* family includes 13 genera and more than 100 phytopathogen species of *Poaceae* family [14]. The genus *Pyricularia* (**Figure 4**), alluding to the piriform shape of its conidia, comprises the fungus *P. oryzae* (teleomorph *Magnaporthe oryzae*) and includes species that are pathogenic to a wide range of monocots [15]. *P. oryzae* is considered a complex and highly variable species, composed by a large number of physiological races or pathotypes, grouped by mating types, having a peculiar phylogenetic characteristics, and host range [17]. In addition, to determine the race of a fungus isolate, the reaction (symptoms) pattern in a set of eight plant cultivars referred to as international differentiators is observed.

Several studies have already pointed to the high genetic instability level of *M. oryzae*, whose genome evolves rapidly in nature. This feature enables the fungus to quickly adapt itself to new selection pressures on the field, often leading to a breaking down of newly launched commercial rice resistant cultivars [18]. This sort of adaptation generally involves genetic mutation and genetic recombination through sexual reproduction, which play important roles in fungus enhancement of genetic diversity [19]. Furthermore, the fusion of mycelia between

strains of opposite mating types leads to sexual spore (ascospores) production, which sometimes culminates in new forms of fungal virulence.

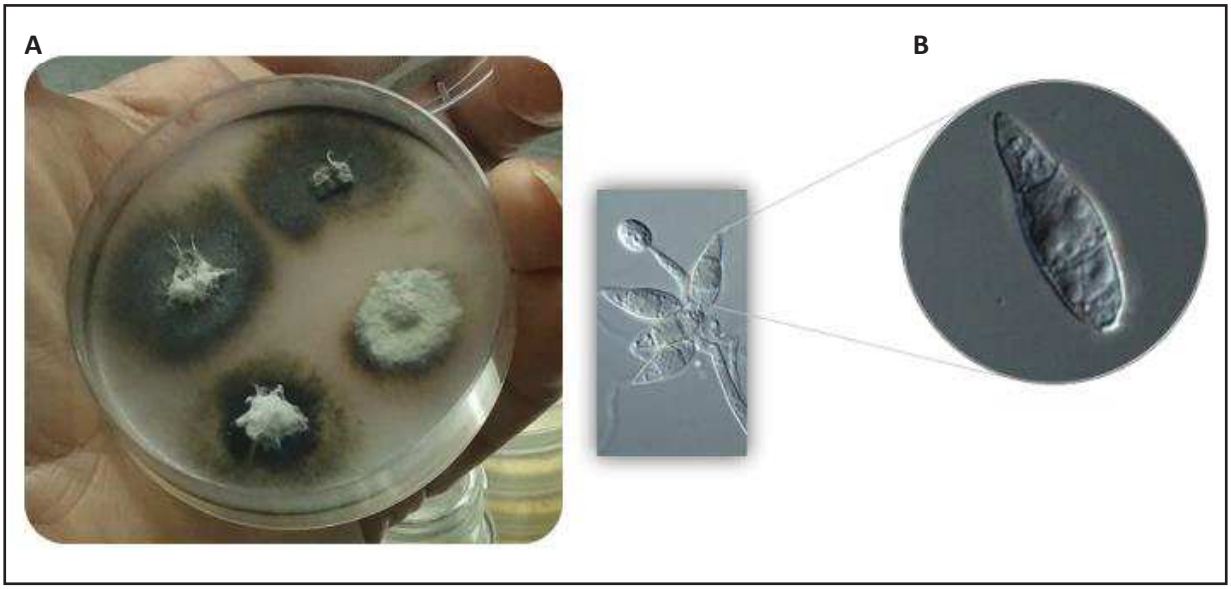

Figure 4. Morphological characteristics of the fungus *P. oryzae*. (A) Image of a growing fungus *M. oryzae* in BDA culture medium showing its fruitification body with 20 days of growth; (B) Micrographs showing the mycelium cellular structure and the development of conidiophores and conidia (Scale bar, 20 μm). In greater magnification, a specimen of a conid, asexual and piriform structure of the fungus *P. oryzae.*

Rice blast poses a major threat to food security worldwide. According to Sharma *et al*.[1], it is estimated that the annual global losses caused by blast would be enough to feed 60 million people. In Brazil, blast occurs in all rice producing states, being notably more relevant in the Midwest and South regions, where losses up 100% in grain yield have been recorded during outbreak cases [20].

M. oryzae infection starts when the conidia (spores produced asexually) are deposited on young rice seedlings followed by germination and *appressorium* formation, structure responsible for disrupting leaf cuticle, culminating in the invasion of plant epidermal cells, as shown in **Figure 5**. The pathogen attacks the leaf and the panicle neck nodule. Infection at the base of panicle, known as neck blast or rotten neck blast, represents the most destructive symptoms in most environments where blast is a problem. [21]. In addition, widespread panicle infections are often lethal for rice yield.

Currently, the most used methods for blast control are the cultivation of resistant varieties, application of fungicide and cultural practices [23]. The latter includes nitrogen fertilization, culture rotation and the use of good quality seeds. The use of resistant cultivars is the most environmental-friendly approach, although only partially controls the disease,

because, as a rule, the resistance conferred by *R*-genes is race-specific. In recent decades, have been sought to breeds that generates greater resistance against *M. oryzae*, leading to the development of rice cultivars that harbor several resistance genes in their genome. However, it seems to be a costly technique in terms of time, labor, and financing [24].

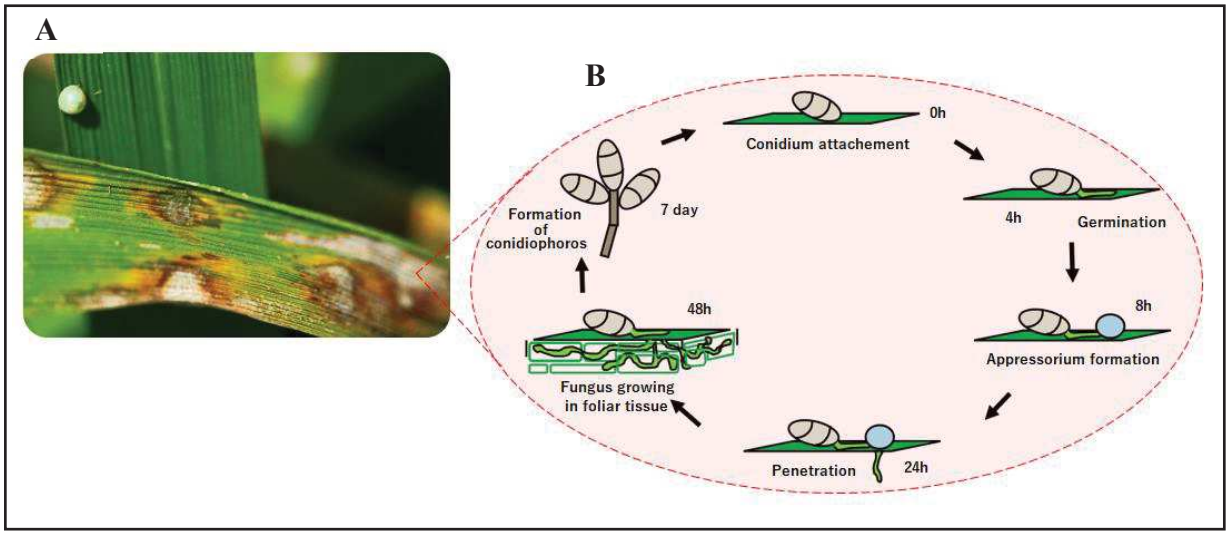

Figure 5. Symptoms of blast disease and the *P. oryzae* infection cycle. (A) Image showing the symptoms outcome of leaf blast; (B) Illustration of *M. oryzae* infection cycle in rice plant, showing the time of each stages of infection. *(Image A, available at http//:knowledgebank.irri.org; Diagram B, adapted from Nadales et al.*[22]*).*

3. Molecular interaction mechanisms of rice-*M. oryzae*

The rice-*M oryzae* interaction at the molecular level has been extensively studied, due in parts to the wide availability of annotated genomic sequences for both organisms (*M. oryzae*: Dean *et al.*,[16]; *Oryza sativa*: Ohyanagi *et al.*[25]). The coevolution between plants and pathogens resulted in a sophisticated plant immune system dedicated to preventing infection and, on the other hand, led to a high degree of adaptation and counter-attack/defense strategies by pathogens. Standing as the front line of plant defense, the detection of pathogen-associated molecular patterns (PAMPs) via recognition receptors located on the cell surface (PRRs), triggers a type of plant immune response called PTI (Pathogen-Triggered Immunity) [26]. As a result, the production of reactive oxygen species (ROS) and the secretion of peptides/antimicrobial compounds in the intercellular space (apoplast) of plant tissue are observed. To defend themselves, specialized pathogens employ virulence proteins (effectors) and other toxins to suppress host defense response [27]. To neutralize the activity of these effectors, plants rely on resistance R-genes ability to directly or indirectly recognize these

effectors, and then activate another elaborate defense system, post-cell invasion, called effector-triggered immunity (ETI).

Therefore, we say that the rice-*M. oryzae* pathosystem is operated by the *gene-for-gene* hypothesis, originally described by H. Flor [28], in which avirulence (*Avr*) genes in the pathogen encode a class of effectors (proteins) or small molecules that alter host's structural functions during infection. When an effector is recognized by the host resistance *R*-gene product, a plant hypersensitivity (HR) response is triggered, preventing pathogen growth through a programmed cell death (PCD) or apoptosis mechanism at the site of infection, often resulting in an incompatible type of interaction (resistance to infection). On the other hand, in a compatible type, the *R*-gene product is not able to recognize the pathogenic Avr effector, resulting in the host's susceptibility to disease (**Figure 6**).

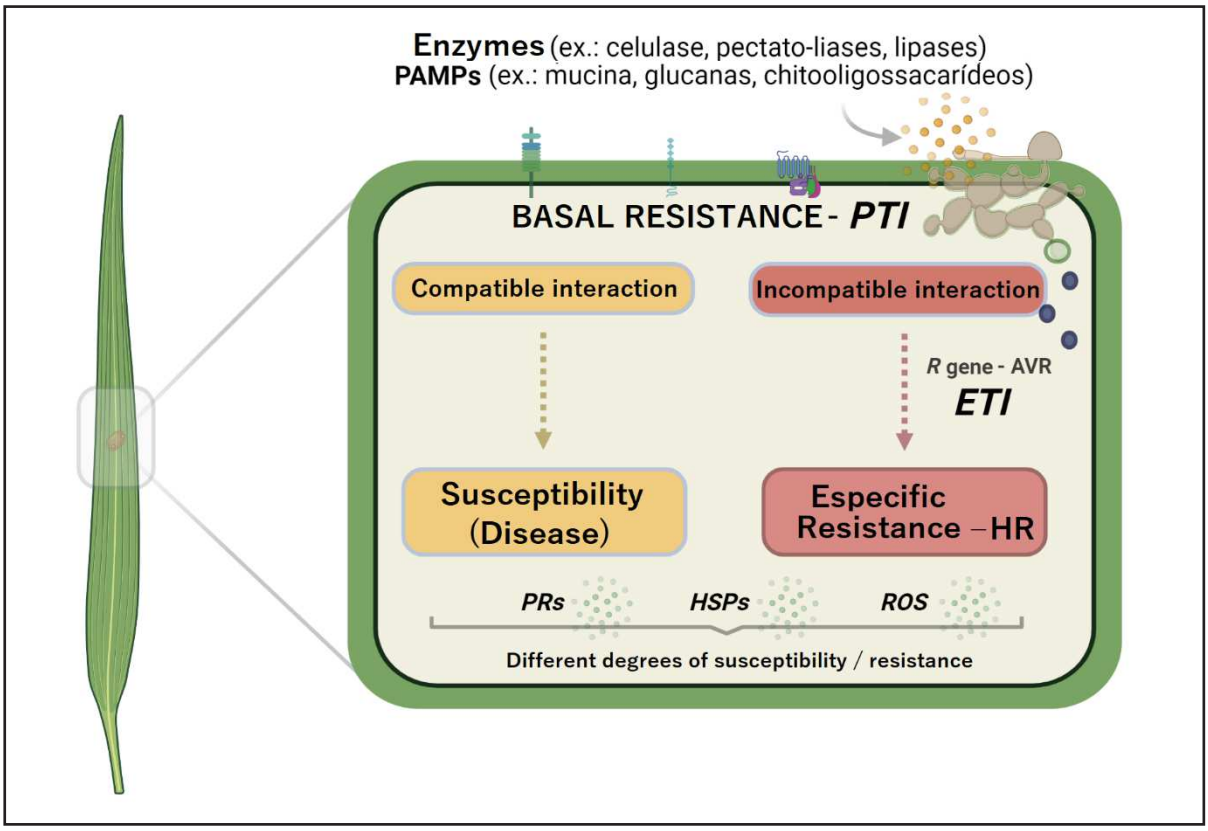

Figure 6. Molecular mechanisms of plant-pathogen interaction. Illustration summarizing the induction of host's main resistance mechanisms upon recognition of pathogen´s effector proteins; the classical types of plant-pathogen interaction (compatible vs. incompatible) and their general outcomes. *PTI, pathogen-triggered immunity; ETI, effector-triggered immunity; AVR, avirulence genes; PRs, pathogenesis-related proteins; HSP, heat-shock proteins; ROS, reactive oxygen species.*

4. *S*-genes: alternative source of resistance beyond plant *R*-genes

In recent years, we have seen a boost in the understanding of how R-genes operate resistance in rice diseases. Currently, more than 100 blast resistance (R) genes (~ 50% in indica, 45% in japonica and 5% in wild species) have been mapped, of which 25 have already been characterized and cloned [29]. However, as mentioned earlier, the plant resistance-building approach employed by conventional breeding programs is negatively influenced by pathogen genetic variability, often resulting in the breakdown of this type of acquired resistance.

As a rule, disease resistance in plants is performed by R-genes showing nucleotide-binding sites (NBS) and leucine-rich (LRR) or protein-serine/threonine kinase (S/TPK) repeat domains [30]. However, in an awkward way, R-genes with NBS-LRR domains act as targets of pathogen effectors, thus playing key roles in host susceptibility. A clear example of this atypical behavior was first reported by Sweat *et al*. [31]. Using *Arabidopsis thaliana* model plant, researchers demonstrated that plant sensitivity to the victorin toxin from *Cochliobolus victoriae* fungus and susceptibility to the disease are conferred by a gene (*Lov1*) that encodes a protein displaying CC-NB- LRR leucine-rich type-domains. In fact, it has been observed that in several other plant diseases caused by necrotrophic fungi, susceptibility is conditioned by a single dominant locus in the host and a cognate toxin derived from the pathogen. For example, the wheat *R*-gene *Tsn1*, encoding a protein having NBS-LRR domains, was reported to be implicated in a response called 'effector-triggered susceptibility' to necrotrophic pathogens [30]. When comparing proteomes of infected and non-infected plant leaves, several classical pathogenesis-related (PR) proteins show an increased abundance in infected foliar epidermis, including peroxidases, chitinases, thaumatin-like PR5 (TLP5) protein. In a very recent study, Lambertucci et al.[32] demonstrated that, against expectations, transient TLP5 gene silencing suggested that TLP5 does not contribute to resistance but rather modulates susceptibility towards *Blumeria graminis*, a fungus that causes *powdery mildew* on grasses, including cereal crops. These observations, ultimately, suggest that compatibility is not just a mere consequence of a failure in host's immune system, but rather that resistance and susceptibility are sides of the same coin, where the genes involved share the same identity and mechanisms of action.

In this context, considering the limitations and/or efficiency of the usage of R-genes to combat blast disease, an alternative strategy that has been used to obtain resistant plants consists in the inactivation of susceptibility genes in the host. Once the disease arises from a compatible interaction, vanishing with a gene in the plant´s genome that plays a critical role in compatibility, can results in a broader and longer-lasting type of resistance. [33].

The term susceptibility gene (or *S*-gene) was first coined by Eckardt [34] to designate genes that confer plant susceptibility to diseases. Genetically, *S*-genes can be defined as being dominant genes, whose loss and/or alteration leads to a recessive resistance [23]. A clear example for this definition is the rice *SWEET* genes, such as *Xa13/Os8N3/SWEET11* and *Os11N3/SWEET14*. Bing Yang *et al*. [35] and Cheng *et al*.,[36] demonstrated the fact that both genetically dominant genes are positively regulated by effector proteins from *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), resulting in susceptibility to this phytopathogen and the disease known as Leaf blight. In contrast, genome the naturally-arose xa13 resistance locus, composed of a series of mutated alleles exists in rice. However, Xa13 resistance occurs only in recessive homozygous mutants and it is due to polymorphisms in the nucleotide sequence of both gene promoter alleles that impairs the induction of rice *SWEET* genes by the TAL (transcription activator-like effector)-type bacterial effector. Although effectors are widely recognized for their role in suppressing host resistance, in fact, several of them act by activating *S*-genes. Thus, rather than suppressing or evading plant immune system, most pathogens, especially hemibiotrophic ones (e.g., *Magnaporthe oryzae* fungus), require host cooperation to establish a compatible type of interaction. Hence, all plant genes that somehow facilitate infection can be considered an *S*-gene [37]. Based on these concepts and in the different phases of host-pathogen interaction, three main molecular mechanisms by which *S*-genes would favor susceptibility were described, thus contributing to disease (I) basic compatibility, in which the expression of *S*-gene helps in recognition, adhesion, and/or pathogen penetration; (II) negative regulation of host immune system, and (III) sustained compatibility (post-invasion), which is necessary for pathogen proliferation, colonization and dispersion [38].

Therefore, *S*-gene inactivation can impair pathogen's ability to cause disease, resulting in longer-lasting and broad-spectrum resistance due to the compromised pre-penetration requirements or insufficient support for specific post-penetration invasion requirements, such as inefficient supply of essential nutrients to the pathogen. One of the most well-known *S*-genes is Mlo (*mildew resistance locus O*), which encodes a membrane protein that acts to support the establishment of haustorium (fungal penetration structure), thus facilitating the invasion of plant epidermal cells [39]. In addition, mlo mutation represents the potential robustness of the strategy, since a recessive mutant in barley (*Hordeum vulgare* L.) that showed resistance to the *Powdery Mildew* (PM) fungus seven decades ago, continues to be employed and still provides durable resistance to all PM races in the field.

Although the understanding of susceptibility´s modus operandi, in its molecular level, is still extremely limited, consecutive discoveries indicate that precise manipulation of host

susceptibility can lead to the development of more effective strategies to fight against diseases, representing an excellent alternative approach to the *R*-genes in breeding programs.

5. Functional Genomics

Functional genomics is a branch of molecular biology that aims basically to integrate knowledge about genome structure, biochemical interactions, molecular functions and gene expression regulation. Its main purposes are to infer about the cause-effect relationship between the observed phenotype and genotype in a given experimental condition[40], evoking biological dynamic aspects, such as regulation of gene expression, transcription, translation, and protein-protein interactions. Usually, researchers employ powerful analytical/experimental tools like transcriptomics, proteomics, gene silencing or gene editing to analyze or explore the generated big data which has greatly contributed, for example, to the prospection of new molecular targets, functional validation and genome editing of candidate genes.

5.1.Transcriptomics and proteomics: tools for the prospection of molecular targets

Transcriptomics consists in comparing gene expression levels under different conditions and stresses [41]. Thus, contrasting genetic materials for a given trait may be analyzed to survey and isolate a specific set of genes responsible for the differential response. Currently, high-performance sequencing platforms, with Next Generation Sequencing (NGS), linked with bioinformatics analysis tools, allow for massive scanning of the genome. For quantitative and qualitative gene expression studies, large-scale sequencing of cell transcriptome is performed from cDNA libraries via RNAseq [42]. In a recent study, Sharma et al. [7] successfully analyzed the transcriptomic profile of rice-*M. oryzae* interaction to capture the molecular basis of broad spectrum *Pi9* blast-resistance rice gene. Using RNA-Seq technology, infected and non-infected resistant lines (with a susceptible genotype serving as a control) were used to survey plant candidate genes engaged with the early rice-blast responses. Authors revealed a sophisticated host cell transcriptional reprogramming during infection. In a recent study [43], transcriptomic sequences were generated from semi-isogenic lines of rice infected with *M. oryzae*. Unlike classic transcriptomics studies, in which a single genotype to compare responses between inoculated and non-inoculated samples are usually employed; or even two genotypes (resistant and susceptible) but presenting different genetic origins, the transcriptomic sequences used here were generated from NILs infected with *M. oryzae*. Developed by the International Rice Research Institute [44], IRBLi-F5 and IRBL5-M NILs, share the same genetic background of Lijiangxin-tuan-heigu (LTH, japonica spp.), a susceptible cultivar to *M. oryzae*, therefore, in

theory, they are identical except for the fact that IRBLi-F5 carries *Pii* and IRBL5-M the *Pi5* resistance gene. These NILs were characterized and displayed different resistance profiles. In the case of IRBLi-F5, the *Pii* gene product does not recognize the *M. oryzae* (isolated 9881 - EMBRAPA microbiological collection, BRM25017) effector protein, resulting in a compatible interaction, while the *Pi5* gene product in IRBL5-M apparently recognizes the effector of the same fungus isolate, leading to a resistance phenotype.

Similarly, proteomics consists of a robust and widespread technique used to analyze cell protein abundance under different biological conditions [45]. The advancement of ionization techniques such as electrospray ionization (ESI), advances in mass spectrometers, and the development of bioinformatics tools have made proteomics a fertile and worldwide spreading field [46]. Proteomics plays a continuum between genome information and the proteome profile in a specific tissue or cell, under different conditions or stresses. Another relevant aspect to bear in mind is the fact that a direct correspondence between transcript levels and the abundance of its corresponding protein does not necessarily occurs, due to several modifying/regulation mechanisms of cell´s machinery.

There are currently very few proteomic studies involving rice-*M. oryzae* interaction, which further reinforces the need of using this technique. Xue et al. [47] employed proteomics to analyze differentially-expressed proteins in rice infected with *M. oryzae*, revealing key enzymes involved in plant innate defense mechanisms. In a more recent proteomic study, Tian et al. [48] analyzed differentially-abundant proteins from interactions between transgenic rice (carrying the Piz-t resistance gene) and virulent and avirulent *M. oryzae* isolates, disclosing complexes protein-protein interactions, tightly involved in rice resistance response. Ultimately, proteomic analysis represents a great source to complement transcriptomics data, leading to a broader picture of biological processes involved in plant-pathogen interaction.

5.2.Antisense technology: gene silencing and in-planta functional validation of target genes

Antisense oligonucleotides are widely used for transient knock-down modulation (post-transcriptional regulation) of gene expression, standing as a crucial tool in functional evaluation of agronomic valuable trait-related genes [49]. The antisense oligonucleotides (ASO) are single-stranded DNA / RNA molecules, synthesized *in vitro*, 13-25 nucleotides long, which may harbor some chemical-modified tags [50]. Represented in **Figure 7**, the concept underlying antisense technology is relatively simple: a short sequence (i.e., oligonucleotide) that hybridizes, according to the Watson-Crick base complementarity concept, to a specific target

mRNA (formation of duplexes), which may act inhibiting its expression and thus blocking the transfer of genetic information from DNA to protein. Regarding the mode of action, two classes of antisense oligonucleotides can be distinguished: (a) oligonucleotides RNase H enzyme-dependent, requiring the activation of this omnipresent enzyme in living beings, which then catalyzes the cleavage (by hydrolysis) of the ASO:target mRNA complex; and (b) steric blocking oligonucleotides, which physically impairs the progression of splicing or translation machinery [51].

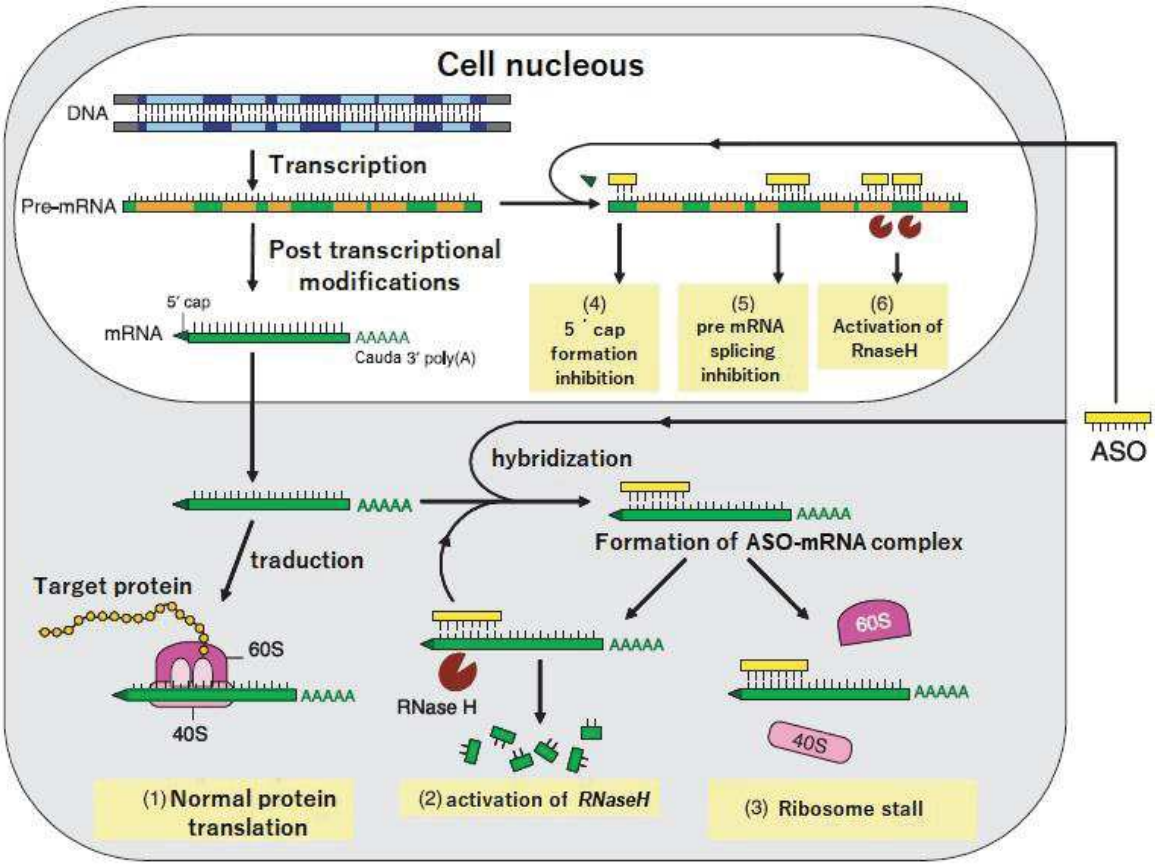

Figure 7. Mode of action of antisense oligonucleotides (ASO). (1) in the absence of ASO, gene transcription and protein translation are maintained. ASO can enter cells by endocytosis and hybridize with target mRNA in the cytoplasm, forming the heteroduplex complex ASO:mRNA which in turn can (2) activate RNase H enzyme, leading to degradation of the target mRNA or (3) interfere with ribosomal assembly by steric blocking. Both (2) and (3) actions may result in the knock-down (decrease) of targeted protein. The binding of ASO to the pre-mRNA (still in the cell nucleus) can negatively act in the regulation of target mRNA maturation through (4) inhibition of 5´cap formation, (5) modulation of RNA splicing, and (6) activation RNaseH *(Adapted from Liao W et. al.*[52]*).*

Although antisense technology is widely applied in gene function analyses, experiments on plants are scarce. One of the major obstacles to the successful use of this tool in plants, precisely concerns its structure stability in intracellular environment, as they tend to be rapidly degraded by intracellular endonuclease enzymes (e.g., phosphatases), usually via its 3´to 5´

nucleasic activity. Notwithstanding, in an interesting experiment carried out by Dinç E. et al. [53], in which antisense phosphorothioate (PS)-oligodeoxynucleotides (PS-ASO, alternatively called as PTO, phosphorothioate oligos) were applied to three different model plants (*Nicotiana benthamiana*, *Arabidopsis thaliana*, and *Triticum aestivum*), authors have addressed relevant concerns about the optimization of delivery, stability and efficiency of oligos in transiently inhibiting the expression of certain genes. Confirming the fact that phosphorothioate (PS) chemical-type of modification considerably increases the intracellular stability of the oligo, the study showed that mRNA and protein levels in the leaves of *A. thaliana* were significantly reduced by up to 85% and 72%, respectively. Furthermore, a recent publication supervised by Bindschedler L. [53], validated the use of PS-modified DNA oligos in silencing *S*-genes. Briefly, they showed thorough PTO-based TIGS (transient-inducible gene silencing) *in planta* assay the precision of antisense oligos not only in modulating *TLP5* (a thaumatin-like protein) transcript levels in the monocot barley (*Hordeum vulgare*) leaves, but also in triggering an increased ROS burst in silenced infected plants, that contributed to disease resistance. Therefore, the usage and new developments of such chemical modifications that improves oligo´s stability and efficacy are of great significance and very welcome in plant biotechnology field.

5.3.CRISPR/Cas9 system for genome editing

The genomic editing technology called Clustered regularly interspaced short palindromic repeats (CRISPR) / Crispr-associated protein (Cas), or simply CRISPR/Cas, is an outstanding approach to the development of plants with enhanced desirable-related agronomic traits. It allows, among several other possibilities, the deletion (knock-out) of genes of interest. CRISPR/Cas is an adaptive immune system present in prokaryotes and initially discovered in bacteria and Archaea [54] and later transformed into a biotechnological tool for gene editing [55].

Unlike previous artificially-engineered enzymes used to manipulate genomes (zinc-finger nucleases and transcription-activator-like effector nucleases), CRISPR/Cas system (outlined in **Figure 8**) is an RNA-programmable gene editing tool, which uses an endonuclease (e.g., Cas9) coupled to a single transcript (guide RNA) to precisely cut any double stranded DNA sequence of interest [55].

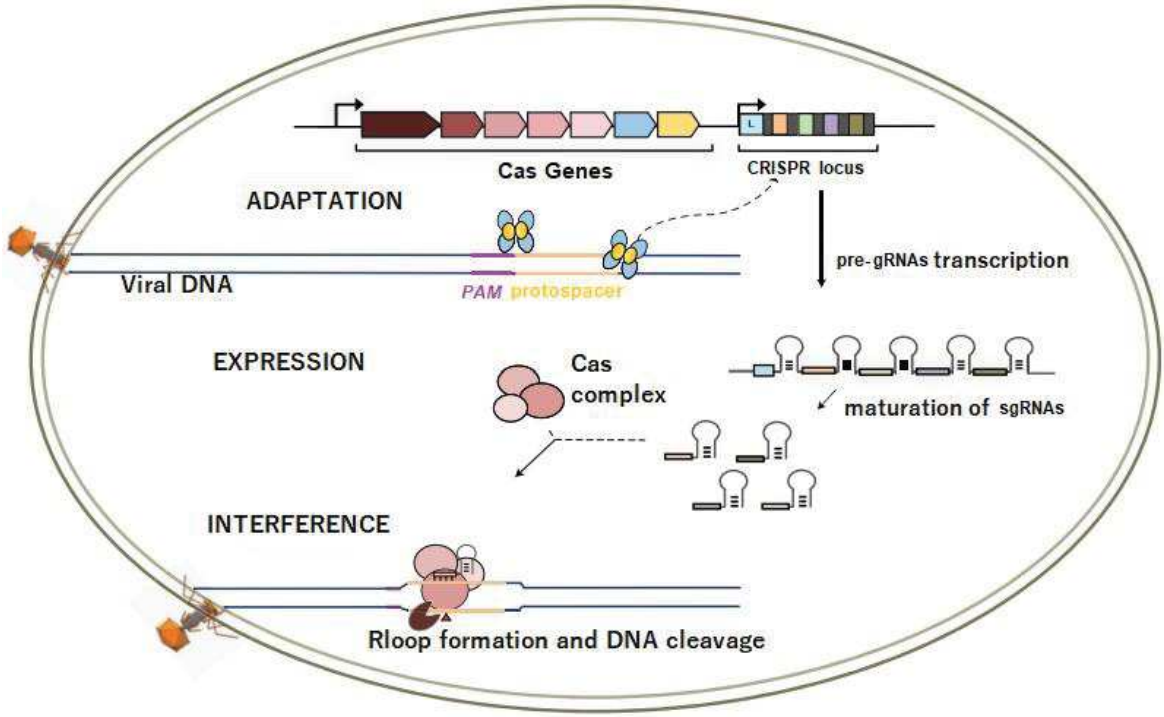

Figure 8. General outline of the three stages of CRISPR/Cas system-mediated immunity. Briefly, during the **adaptation** phase, a complex of Cas enzymes selects a specific part of the exogenous DNA and integrates it into the CRISPR locus in host genome. In the next stage (**expression**), the CRISPR array is transcribed in pre-gRNA which is further processed by other Cas proteins. In the last phase (**interference**), mature gRNAs now coupled with endogenous nucleases (e.g., Cas9) interrogates all foreign DNA, searching for a DNA stretch that matches (by sequence homology) with the guide-sequence, and also a PAM (*protospacer adjacent motif*) sequence. Once found, the intruder DNA is immediately cleaved *(Adapted from Charpentier et al.. [56]).*

Generally, CRISPR/Cas system generates a break in the double strand of targeted DNA, known as a "double-strand break" (DSB), triggering the endogenous cell repair machinery, that encloses the two most known types of damage repair systems: Non-Homologous End-Joining (NHEJ) and Homology-Directed Repair (HDR). The NHEJ type repair, prevalent in the plant kingdom, is highly error prone and often introduces small deletions and/or insertions (Indels) at the junction of the newly repaired double strand of DNA. If such Indels interfere with the codon reading frame (for example, causing a frameshift mutation) or generates a stop-codon in the targeted gene product, a knockout (loss-of-function mutation) is created (**Figure 9**).

Such discovery immediately paved the way for new opportunities in science, including the possibility of understanding very quickly several plant biological systems. Based on a recent bibliographic search on the PubMed - NCBI platform (http://ncbi.nlm.nih.gov), the main uses of CRISPR system in agriculture have been focused on the improvement of yield performance, biofortification, tolerance to biotic and abiotic stress, with rice (*Oryza sativa* L.) being the most

studied crop. Although, most research articles involving CRISPR/Cas system application in agribusiness commodities have been made just as proof-of-concept studies [58].

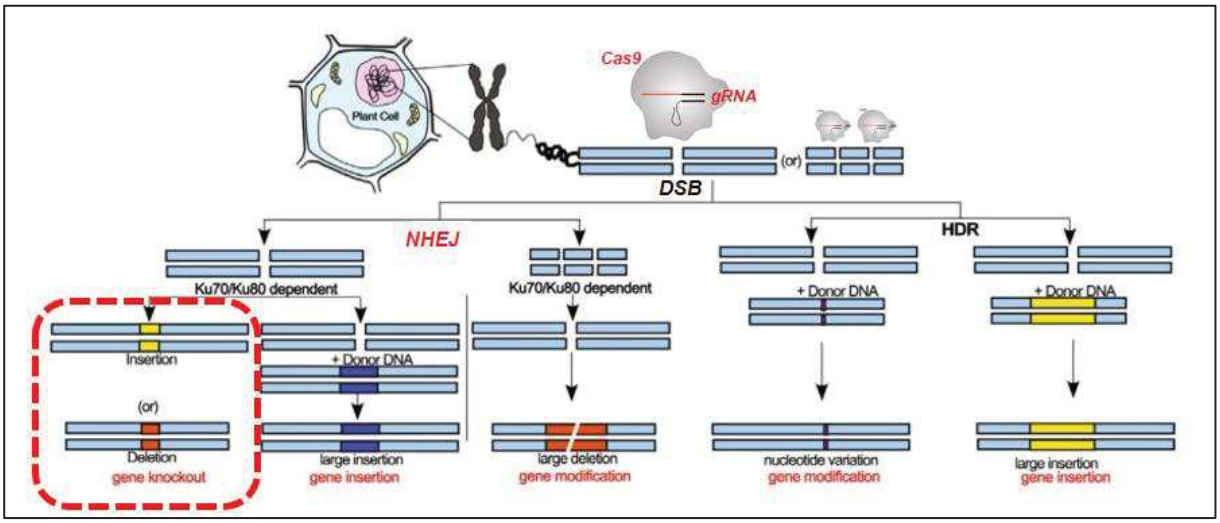

Figure 9. General scheme of the DNA repair process in a plant cell. Right after specific breaks in the double-stranded DNA induced by RNA-guided nucleasic enzymes (e.g., Cas9), basically two types of repair pathways are stimulated: NHEJ- non-homologous end-joining and HDR- homology directed repair. Dashed red frame highlights two possible outcomes of NHEJ repair pathway, in which insertion (yellow) or deletion (orange) type of mutations may lead to the gene silencing *(Adapted from Subburaj et al ..[57]).*

Although some potential blast susceptibility genes have already been revealed and validated through RNAi silencing or overexpression studies [59], [60], researches using CRISPR/Cas system to increase rice-blast resistance are still very scarce. In a pioneering study, conducted by F. Wang et al. [61], CRISPR/Cas9 system was used to delete the OsERF922 gene in rice spp. japonica. According to literature, plant factors responsive to ethylene (ERF), members of the APETELA2/ERF subfamily and of the transcription factor (TF) superfamily in plants, these genes are involved in the modulation of multiple abiotic and biotic stresses tolerance [62]. These authors revealed a considerable decrease in the number and length of blast lesions in all mutant lines infected with *M. oryzae*, compared to the uninfected control. Moreover, they did not detect any significant pleiotropic effects in agronomic-related traits, such as plant height and grain weight, between T2 mutant lines and wild type plants. These findings remarkably contribute to the ever-expanding repertoire of rice *S*-genes, bringing relevant implications for engineering resistance in plants, enabling the launch of new commercial elite cultivars.

5.3.1 Social-economics and regulatory perspectives

Considering the technical and political issues and public acceptance of CRISPR/Cas usage in agriculture, we have the heritability of induced-mutation and the generation of transgene-free plants representing the two major concerns. Fortunately, some studies have reported the induction and stable inheritance of single [63] and multiple [64], [65] mutations, when evaluating T0 plants and up to T3 progenies. In general, the mutants of interest are selected by the mechanism of genetic segregation via Selfing or backcross breeding techniques [66]. To address the heritability concern, Gao et al. [67] used an interesting visual tool to demonstrate the inheritance of mutations induced in the *pds* gene of *O. sativa*. The *OsPDS* gene encodes the phytoene desaturase enzyme, which plays a key role in carotenoid biosynthesis. Silencing this gene causes photodegradation or albino phenotypes. Thus, the authors were able to monitor patterns of mutation and inheritance with a visual indicator, associated with genotyping and sequencing. These authors demonstrated that the CRISPR/Cas system can induce hereditary mutations in rice plants from T0 to T2 generations and that homozygous and bi-allelic mutants were generated still in the first generation. By the way, we will revisit this subject in more details soon at the first chapter of the present thesis, more precisely at the topic '*perspectives on the horizon*' on the published review.

In Brazil, according to the Normative Resolution No. 16 of the National Technical Commission on Biosafety – CTNBio [68], plants generated by CRISPR genome editing technology tend to be treated as non-GMO. However, in order to generate transgenic-free plants, it is necessary to obtain stable CRISPR/Cas induced-mutated plants, without the presence of any DNA expression cassette inserted in the host genome.

Most studies using Agrobacterium-mediated transformation aim to generate mutant plants without the transfer DNA cassette (T-DNA) through the genetic segregation of the transgene. At the end of the transformation process (e.g., plant regeneration step) researchers often screen mutants for the presence of transgenes using specific primer pairs to amplify vector-containing genes encoding (e.g., Cas nuclease), or any selective marker gene (e.g., hygromycin). Other studies, using a more sophisticated CRISPR strategy have demonstrated the viability in generating transgenic-free plants using alternative methods to deliver CRISPR DNA-free components, thus avoiding the presence of any exogenous DNA in the whole process[69][70][71]. This can be achieved by different approaches, such as transfecting protoplasts with a ribonucleoprotein complex, as already demonstrated in different cultures, including rice[72], [73] (here, the regeneration of entire plant from protoplast cells is still a huge bottom neck for most of crops), or using particle bombardment method to deliver CRISPR-IVT(-*in vitro transcribed*) reagent directly to somatic-embryogenic calli tissues of

plants [74]. As evidenced above, several studies have demonstrated the heritability and transgene-free characteristic of crop plants generated by CRISPR gene editing, records that uphold the safety of CRISPR technology usage and its potential applications in agribusiness.

Aims of the thesis

The main objective of this work was to develop rice plants resistant to the fungus *Magnaporthe oryzae* by the knockout of plant susceptibility genes through the application of CRISPR/Cas9 system. To achieve this challenge, four specific goals were proposed:

I. Initially, a review in CRISPR/Cas genome editing technology was proposed in order to gather general information about the tool, enclosing the up to date discoveries and their applications in agribusiness, stressing its role in the improvement of major agricultural crops, including rice;

II. Facing the scarce number of susceptibility genes described in the literature and experimentally validated in rice-*M. oryzae* pathosystem, the second objective was to perform a shotgun proteomics analysis for the identification of specific proteins potentially associated with host's susceptibility, upon the interaction of rice near-isogenic lines (NILs) with the fungus *M. oryzae*. A better understanding of expression profiles of differentially-abundant proteins between compatible (susceptibility) and incompatible (resistance) interactions was, ultimately, essential to reveal potential targets and also their roles in host susceptibility;

III. The third goal was to carry out a functional validation *in planta* of the identified potential S-genes via Transient-Inducible Gene Silencing (TIGS) approach, using antisense oligonucleotides (ASO);

IV. The fourth and most challenging objective was the rice genome editing using CRISPR/Cas9 system to knockout potential susceptibility genes aiming at the development of blast resistant rice lines.

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Chapter I:

CRISPR Genome Editing Technology: A Powerful Tool Applied to Developing Agribusiness

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CRISPR Genome Editing Technology: A Powerful Tool Applied to Developing Agribusiness

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Abstract

The increase in human population causes an increasing demand for food. In the current non-optimistic global scenario, where adverse climate changes come associated with substantial population growth, the main challenge in agribusiness is food security. Recently, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas system has emerged as a friendly gene editing biotechnological tool, enabling the manipulation of novel traits in several organisms. This review addresses the role of CRISPR/Cas system as a paramount tool for the improvement of agribusiness products. Here, we highlight recent findings and potential applications of CRISPR/Cas genome editing technology in improving major traits in agricultural plants, farm animals and fish of economic importance. Agriculture, livestock, and aquaculture commodities represented mainly by plant crops, farm animals, and fish, respectively, are the natural resources responsible for feeding the world. CRISPR/Cas studies from the bench to applied research in the field, promotes trait improvements of agricultural, livestock and aquaculture products, including animal productivity and welfare, plant crop yield and quality with substantial positive impacts in human health and nature. CRISPR/Cas system has revolutionized bioscience and biotechnology, and its concrete application in agribusiness goods is on the horizon.

Keywords: *Genome editing; Food production; Commodities; Agriculture; Livestock; Aquaculture;*

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1. Introduction

In general, agribusiness represents a dynamic and strategic global economic sector, responsible for the production, distribution, commercialization and use of food, feed, fiber, forest products and fuels [1]. According to the FAO and USDA, the major agribusiness products (Table 1) are agricultural commodities, such as sugar cane, maize, rice, soybean and wheat, while the main livestock commodities include dairy products, as meat (from pigs, chickens, and cattle), and eggs [2-4]. Fishery products also figure in this list and include fish, crustaceans, mollusks and others. In the current non-optimistic global scenario, where adverse climate changes come associated with substantial population growth estimated to escalate to ten billion people by the end of 2050, food security represents the major challenge to be faced worldwide [5]. Therefore, broaden the application of innovative biotechnological outputs on the agribusiness field is vital to respond the increase in food demand, ensuring a sustainable production.

Table 1. Worldwide production of main commodities in 2018.

Worldwide production (2018)	
Commodities	Production (tons)†
Milk	6,832,117,056
Sugar Cane	1,907,024,730
Maize	1,147,621,938
Rice	782,000,147
Wheat	734,045,174
Soybean	348,712,311
Fishing products/ Aquaculture production	148,474,349
Meat (pig)	120,313,264
Meat (chicken)	114,266,750
Eggs (chicken)	76,351,425
Meat (cattle)	67,353,900

† According FAO (2020).

In this context, Clustered Regularly Interspaced Short Palindromic Repeats/Cas (CRISPR-associated), easily called CRISPR/Cas system, stands as a breakthrough genome editing technology with countless potential applications to improve agribusiness performance worldwide [5, 6] (**Figure 1**). The first experimental evidence of its biological role of CRISPR/Cas system was determined only in 2007 [7]. The CRISPR/Cas system, basically composed by a CRISPR locus and *cas* genes organized in operon, represents an adaptive immune system present in bacteria and archaea domains that confers resistance against phages and most foreigner genetic elements by chopping intruder´s nucleic acid genome [8]. CRISPR/Cas systems encompass 2 classes, 6 types and 33 subtypes (for a more detailed review of this topic, see [9, 10]. However, the CRISPR-Cas system thoroughly described and widely employed in plant genome editing is an adaptation of type II CRISPR-Cas system (**Figure 2**) found in *Streptococcus pyogenes* [11, 12].

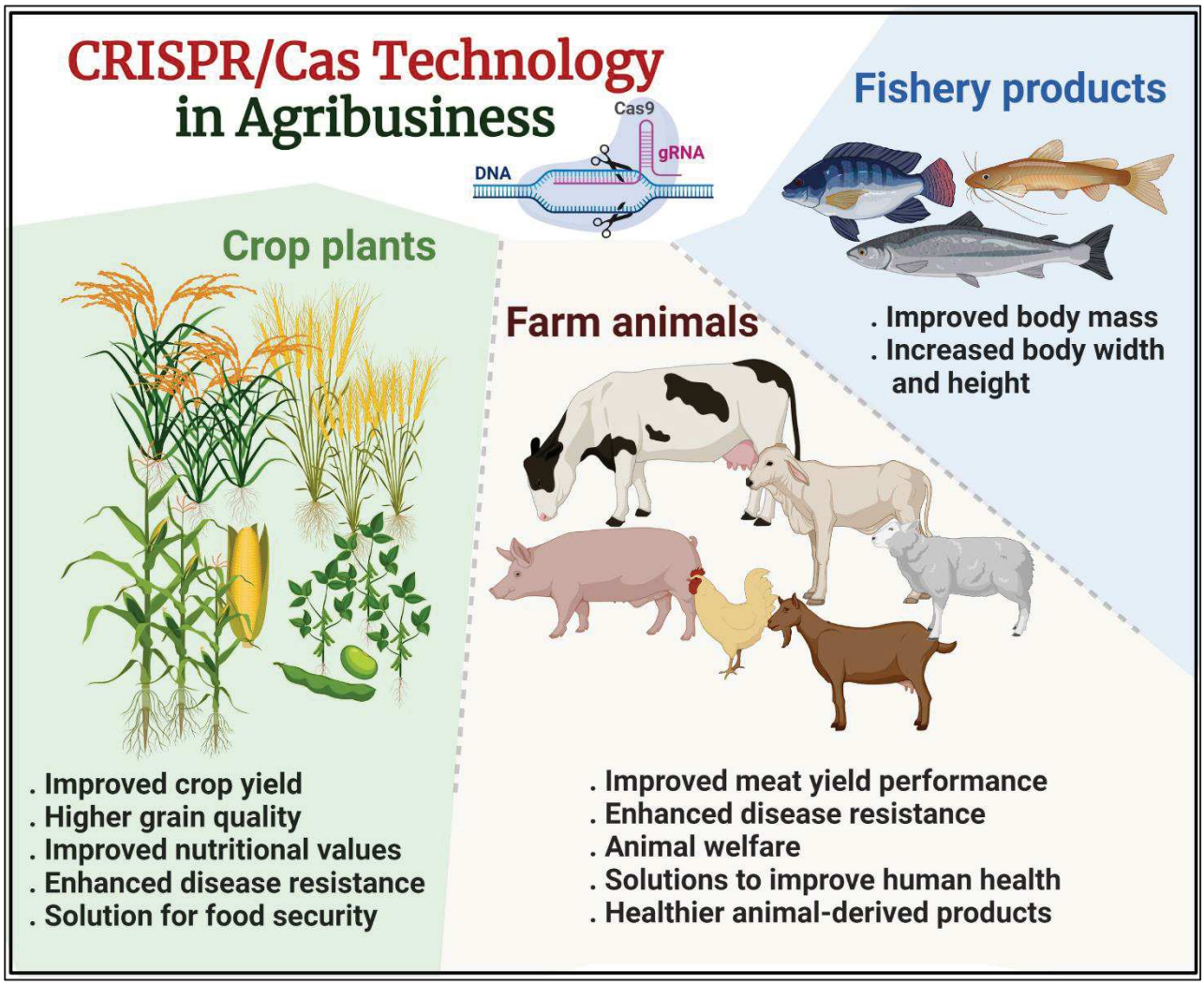

Figure 1. General applications of CRISPR/Cas genome editing technology.

This system is dependent on the guide RNA (gRNA) sequence specificity of at least 10 base pairs (seed sequence) at the 3'-end of the gRNA. For an efficient on-target cleavage by Cas9 nuclease the existence of a protospacer adjacent motif (PAM), immediately downstream of target DNA sequence is mandatory [12, 13]. These PAM sequences, preserved in foreign genomes, are crucial for host recognition of self versus non-self genetic material [8, 14]. Cas9 endonuclease from *S. pyogenes* specifically recognizes NGG nucleotide sequence as a PAM, and presents two catalytic nuclease domains: RuvC and HNH (Figure 3), which cut the PAM- containing strand and its complementary strand (target DNA sequence strand), respectively, thus generating a double-strand break (DSB) that abrogates infection [15]. Furthermore, the formation of DSB at the desired target sites with colossal precision are extremely desirable in functional genomics, allowing gene disruption, creation of precise point mutations, and knock-in of DNA sequences basically by two host cellular DNA repair routes: NHEJ - non-homologous end joining and HDR - homology-direct repair (HDR) [16] (**Figure 3**).

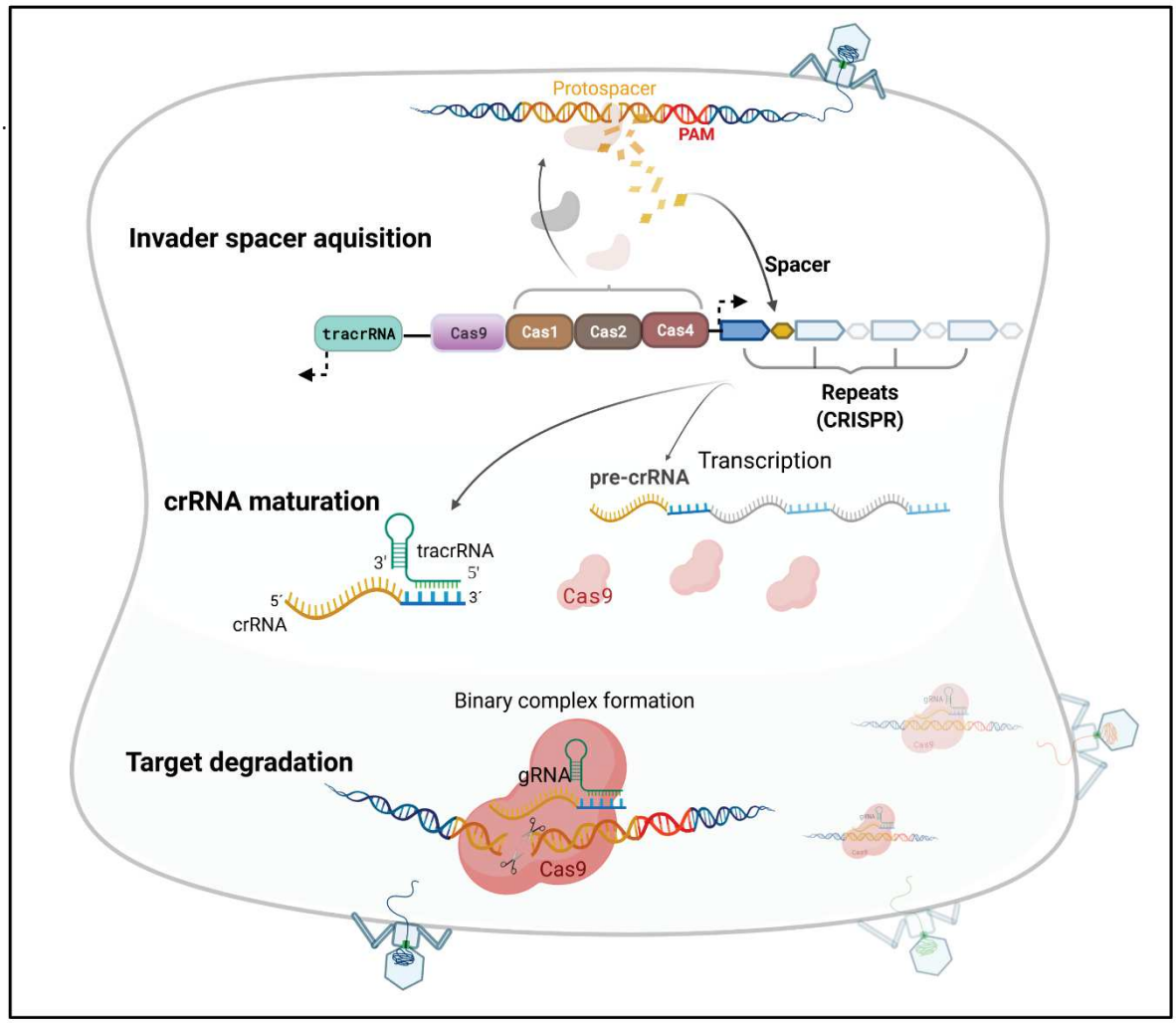

Figure 2. Overview of type II CRISPR–Cas bacterial adaptive immunity mode of action. Upon entry of foreign genetic elements (in this case, a viral DNA) in the bacterial cell, a native Type II CRISPR-Cas system is turned on. At the first meet (invader spacer acquisition), CRISPR-Cas associated proteins (Cas1, Cas2, and Cas4) select and process a specific part of the invading DNA (protospacer) and integrates it as a new spacer into a CRISPR locus. After that, the CRISPR array including invader-derived spacers is transcribed as a pre-crRNA that bounds to tracrRNA transcripts, and is processed into guide-RNAs (crRNA maturation). The guide-RNA forms a binary complex with Cas9 enzyme that further scans invading DNA searching for a PAM sequence adjacent to a 20-nt spacer complementary sequence to chop it (Target degradation), generating a double-strand break (represented by scissors) that neutralizes the invader.

In this review, we focus on the up-to-date applications of CRISPR/Cas system in the improvement of specific agribusiness commodities, such as major agricultural crops, including rice, maize, wheat and soybean, as well as livestock (milk and meat), and aquaculture (fishery products). The limitations, future challenges, and ethical concerns of CRISPR-Cas technology applied to agribusiness are also discussed.

2. CRISPR/CAS in major commodities

In the past thirty years, genetic engineering has revolutionized agribusiness by enabling genome improvement of commercially-interesting targets. Recently, CRISPR/Cas system has been in evidence due to its robustness and versatility, allowing performing diverse gene editing strategies [17]. This breakthrough technique is a helping hand for researchers in crop domestication and development of ideal plants and animals with improved traits, i.e., high yield, improved quality and abiotic/biotic stresses tolerance [17, 18].

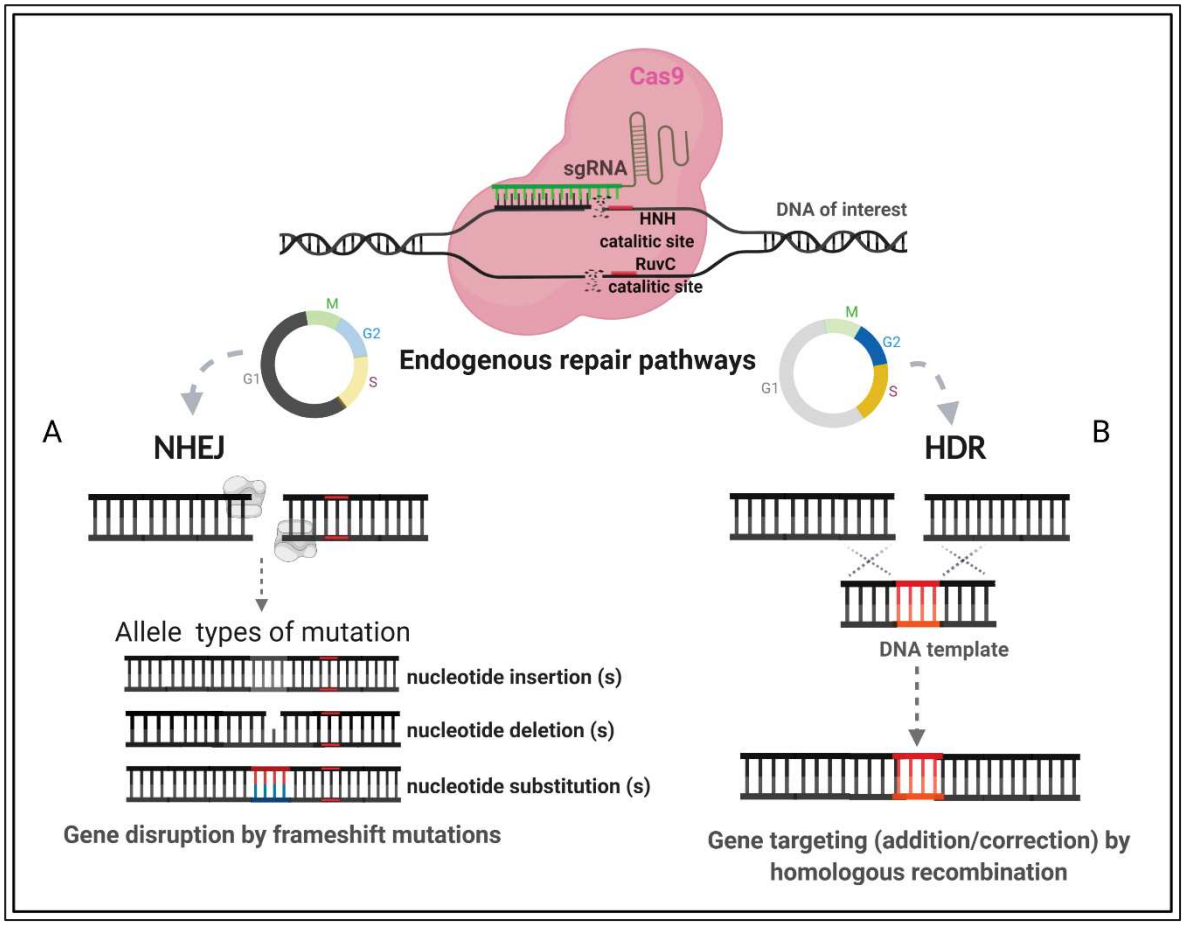

Figure 3. Outcomes of CRISPR–Cas9 system-mediated genome editing. User-tailored single guide RNA (sgRNA) drives the Cas9 enzyme to a specific DNA target site and a double-strand break (DSB) is generated disrupting both the PAM- containing strand and its complementary strand (target sequence strand). Depending on the cell cycle phase, genomic lesions can be fixed basically by two distinct DNA repairing routes. As a rule, the error-prone non-homologous end joining (NHEJ) repair pathway takes place and mono/bi-allelic insertions, deletions, and substitutions of nucleotides are the common outcomes, resulting in a frame shift mutation, hence the loss of gene function. Alternatively, error-free homology-directed repair (HDR) route can drive genome repair and by homologous recombination insert a given donor template sequence, enabling to perform knock-in and gene correction strategies.

3. CRISPR/Cas in rice: focusing on yield and quality boost

Rice (*Oryza sativa* L.) is one of the most important food sources in the world [19]. The ongoing context of threatened food security makes rice a strategic crop and its yield and quality traits main targets for improvement via CRISPR/Cas technology. Several morpho-physiological

features of rice plants are known to have a direct or indirect impact on rice yields, including plant height, grain weight, number of grains per panicle, number of panicles per plant, and tiller number [20, 21]. So far, gene knock-out strategy is one of the most straightforward CRISPR/Cas applications, and (**Figure 3**) has proven to be highly efficient for rice productivity improvement, with interesting phenotypes associated with loss-of-function mutations unraveling potential genes engaged with positive/negative regulation of yield-related traits (table 2).

Liu, et al. [22] employed CRISPR/Cas9 to edit rice cv. kasalath (presenting an *indica* spp. genetic background) and reported a substantial increase of grain width and weight by simply knocking out the *OsGW5* (*grain weight* 5) gene. Another relevant study conducted by Li, et al. [23] successfully applied the CRISPR/Cas9 simplex system to knock out four rice genes, *OsGn1a*, *OsDep1*, *OsGs3*, and *OsIpa1*, previously claimed to act as negative regulators of rice grain number, panicle architecture, grain size, and plant architecture, respectively. They reported an enhancement of panicles, grain number and size, positively impacting rice yield-related traits.

Another CRISPR/Cas9 simplex function analysis study [24] revealed a so far unclear genetic network controlling grain size. It was demonstrated that the locus *GLW2* (*grain length and width* 2), a dominant QTL, positively regulates rice grain weight throughout the expression of *OsGRF*4 (*growth-regulating factor* 4) locus-encoded gene, which is negatively regulated as a rule by the microRNA's mechanism. The generated CRISPR/Cas9 mutations in the OsGRF4 target gene were able to change the detection by miR396C, which resulted in an improvement in grain size and production. Substantial improvements in rice grain yield were also achieved by Lu, et al. [25]. Notwithstanding the role of amino acid transporters to allocate nutrients throughout plant cells during its life cycle, these authors demonstrated that CRISPR/Cas9 knock-out of *OsAAP3* (*amino acid permease* 3) gene in rice ssp. *japonica* enhanced rice grain yield.

The CRISPR/Cas system has also proved its potential and feasibility to improve rice yield by targeting plant architecture-related traits. Using a similar strategy to target negative regulators of important yield-related pathways, Butt, et al. [26] successfully disrupted the *OsCDD7* (*carotenoid cleavage dioxygenase* 7) gene, which controls the biosynthesis of strigolactones, which are plant hormones essential for plant architecture, such as the inhibition of branching [27]. Edited plants presented an increase in number of tillers that resulted in a substantial improvement in rice yield. Notwithstanding the aforementioned pieces of evidence, plant breeders argue that due to complexity of most yield-related traits, usually controlled by QTL and influenced by external environmental determinants, simply knocking out individual factors may not be sufficient to improve rice yield [21, 26, 28]. Xu, et al. [29] used a CRISPR/Cas multiplex strategy to simultaneously edit three well-characterized rice QTLs related to grain weight (*OsGW2*, *OsGW5*, *OsTGW6*). They reported an additive effect in triple mutants displaying substantial increase in seed weight, length, and width when compared with double mutant phenotypes. Similar gene pyramiding additive effect results favoring rice productivity were also obtained by J. Zhou et al. (2019). [28] These authors employed the CRISPR multiplex strategy to generate several combinations of double and triple knockouts of genes related to yield, including *OsGS3*, *OsGn1a*, and *OsGW2*, well known to negatively regulate grain size, number, and weight, respectively.

Crop growth and yield are also under strong regulation of plant hormone complex interactions [30]. Abscisic acid (ABA) phytohormone, for example, plays key roles in plant growth and stress responses. Miao, et al. [31] performed for the first time a CRISPR/Cas multiplex strategy to target an entire rice family of abscisic acid (ABA) receptors, known as pyrabactin resistance 1 (PYR1)/PYR1-like (PYL). Several combinations of knocked-out mutants for *OsPYL* genes successfully resulted in different degrees of rice growth and improved grain productivity. Representing a major source of nutrients in the daily diet of billions of people around the world [19], food security involves not only yield, but also grain quality. To date, the CRISPR/Cas system has also been applied to exploring several quality-related traits, generating new rice varieties with

improved quality-associated features, such as appearance, fragrance, cooking-eating, and nutritional values,[32] the last having a special appeal in developing countries facing an overall

Table 2. Characterization of CRISPR/Cas strategies used for improvement of rice (*Oryza sativa* L.).

Commodity	CRISPR nuclease	Delivery system	CRISPR mechanism	Transfection method	Aim	Explant/ Cell type	Target	Targeting efficiency	Reference
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Improvement of grain yield	Mature embryo	*OsGW5*	No data	[81]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Improvement of grain yield	Mature embryo	*OsGn1a* OsDEP1 OsGS3 OsIPA1	42.5% 67.5% 57.5% 27.5%	[23]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Determination of a regulatory network controlling grain size	Mature embryo	*OsGRF4*	No data	[24]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Improvement of grain yield	Mature embryo	*Os4AP3*	No data	[25]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Engineer plant architecture to improve yield	Mature embryo	*OsCCD7*	No data	[26]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Simplex/Multiplex Knock-out	*Agrobacterium*	Improvement of grain yield	Mature embryo	*OsGS3* *OsGW2* *OsGn1A* *Triple mutant*	12.8% 15.1% 13.86% 12.80%	[28]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Multiplex Knock-out	*Agrobacterium*	Improvement of grain weight	Mature embryo	*OsGW2* *OsGW5* *OsTGW6*	No data	[29]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Improvement of quality	Mature embryo	*OsSBI* *OsSBIIb*	40% 26.7%	[35]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Improvement of quality	Mature embryo	*OsWax*	83% (XS134) 87% (9522)	[38]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Improvement of quality	Mature embryo	OsFAD2-1	No data	[39]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Improvement of quality	Mature embryo	*OsNramp5*	70 – 82.4%	[100]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Improvement of quality	Mature embryo	*OsBadh2*	No data	[101]
Rice (*Oryza sativa* L.)	Cas9	Plasmid	Simplex/Multiplex Knock-out	*Agrobacterium*	Improvement of distribution and production	Mature embryo	*Os.Hd2* *Os.Hd4* *Os.Hd5*	77.8%	[102]

vitamin deficiency in their daily diet [33]. Having a singular positive impact in public health, a fine-tune adjustment of rice grain nutritional properties could bring health benefits to patients with diet-related chronic diseases [34]. Aiming to develop a rice variety with an improved content of resistant starch (RS), a specific type of starch beneficial for human health, Sun, et al. [35] used the CRISPR/Cas9 system to successfully generate high-amylose and improved RS rice by targeting *OsSBEI* and *OsSBEIIb* (*starch branching enzyme*) genes. OsSBEIIb knock-out mutants showed up to a 9.8% substantial increase of the desired resistant starch content. Interestingly, amylose is argued to be the most important cooking-eating quality beacon [36].

However, for some rice varieties, mostly the *indica* type, the high amounts of amylose represent wide market constraints [37]. Being a monogenic-controlled trait, CRISPR/Cas9 was successfully performed and loss-of-function mutants (waxy gene knockouts), exhibited a significant reduction of amylose content (85% lower in the comparison with the WT), reaching desirable and ideal levels for commercialization [38].

Likewise, aiming to improve health benefits and also suppress lifestyle diseases, Abe, et al. [39] employed CRISPR/Cas system to improve rice grain fatty acid composition and generate a healthier oleic acid. One of the three functional *fatty acid desaturase 2* genes (*OsFAD2-1*) was selected as target. The loss-of-function mutant rice plants showed higher levels of oleic acid, with at least a two-fold increase compared to WT non-transformed plants. Therefore, the CRISPR/Cas-based genome editing tool already stands as a cost-effective strategy to target complexities of most yield and quality-related traits toward the development of improved rice varieties worldwide.

4. *Precision breeding in maize*

Maize (*Zea mays* L.) is high important in human and animal diet, and perform an important role in studies concerning gene function and inheritance in plant development, is an important model organism for genetic research. It has become a staple food worldwide, with other applications involving biofuel production, as well as production of animal feed and compounds for the industry in general.

Liang, et al. [40] were the first researchers to applied CRISPR/Cas system for editing the genome of maize based on the nuclease SpCas9. A PEG-mediated transformation protocol was used for the transfection of protoplasts with plasmids harboring Cas9/gRNA genes in order to evaluate knock-out effects for the *ZmIPK* gene, related to the biosynthesis of the antinutrient phytic acid, by using two gRNA sequences to target different regions of *ZmIPK*. This was the first report on the applicability not only for CRISPR/Cas in maize, but also for TALEN, in which they showed similar results for both editing systems regarding targeting efficiency.

Hence, maize can be appointed as one of the first crop to be edited by using CRISPR technology for precise genome mutagenesis. Later, Svitashev, et al. [41] used a different strategy for the transfection of plasmids harboring Cas9/gRNA genes, based on the particle bombardment (biolistic or gene gun) of immature embryos. This work aimed to demonstrate the suitability of Cas9 for plant breeding by trying different approaches, including knock-out and knock-in. Hence, the researchers selected as target both CDS (exons) and promoter (5'-UTR) sequences.

Thus, since 2016 many other researchers have performed gene editing in maize with CRISPR/Cas systems by different strategies (table 3). However, regardless the CRISPR employed mechanism, explants used for maize transformation are mostly immature embryos, since protocols using embryo cells are very well established. To deliver CRISPR reagents into immature embryos, *Agrobacterium* and biolistic-mediated transformation protocols are frequently employed. Some authors have also performed gene editing using isolated protoplasts with PEG-mediated transformation. No other transformation strategies have been reported for genome editing in maize so far.

Most of published articles report the use of DNA plasmids to load Cas/gRNA-coding genes. However, a less frequent but not less efficient strategy has also been reported, based on a plasmid-free Cas/gRNA ribonucleoprotein (RNP) complex. In this strategy, microparticles are adsorbed with pre-assembled RNP complexes (Cas purified protein and gRNA transcribed *in vitro*, mixed in a specific molar ratio) and transfected through biolistics likewise used for gene constructs, bypassing the drawback represented by transgene integration and further requirement of Mendelian segregation phases to obtain transgene-free plants. Svitashev, et al. [42] were the first to use RNPs and to demonstrate their feasibility in maize gene editing. Next, Young, et al. [43] also used RNPs in parallel to plasmids to independently evaluate and validate off-target activity by targeting *lig1*, *Ms26* and *Ms45* genes, located in different chromosomes.

Although the majority of CRISPR experiments have employed Cas9 nuclease, more recently Lee, et al. [44] used both Cas9 and the Cas12a (*Cpf1*) to compare on-target activity in maize. The authors aimed to knock-out the *glossy2* (*Zmgl2*) gene, responsible for the epicuticular wax formation in young leaves, and thus with easy phenotype characterization. Unfortunately, Cas12a showed a remarkable low targeting efficiency (0 to 60%) compared to Cas9 (90 to 100%). Several other authors reported the use of plasmid systems with distinct CRISPR approaches. Feng, et al. [45] performed a knock-out of the *Zmzb7* gene that encodes for IspH protein from the methyl-D-erythritol-4-phosphate (MEP) pathway, to evaluate its potential as a marker targeting. Feng, et al. [46] also performed the knock-out of the same gene along with *Zmzyp1* and *Zmsmc3* genes, reported to encode the main protein of the synaptonemal complex and a protein hypothetically involved in chromosome structural maintenance, respectively. The authors obtained edited plants for the three loci with a targeting efficiency of about 66% by successfully using *dmc1* and U3 promoters of maize for Cas9 and sgRNAs expression, respectively.

Table 3. Characterization of CRISPR/Cas strategies used for improvement of maize (*Zea mays* L.).

Commodity	CRISPR nuclease	Delivery system	CRISPR mechanism	Transfection method	Aim	Explant/ Cell type	Target	Targeting efficiency	Reference
Maize (*Zea mays* L.)	Cas9	Plasmid	Knock-in	Biolistics	Improvement of grain yield under drought stress	Immature embryo	5'-UTR of *ARGOS8*	60-98%	[47]
Maize (*Zea mays* L.)	Cas9	Plasmid	Knock-in	Biolistics *Agrobacterium*	Generation of CTL for multiple transgenic traits	Embryo Immature embryo	*Bbm* *Wus2* *FLP* *nptII* and *pmi*	8-99%	[50]

CTL: Complex Trait Loci; FLP: flippase recombinase.

Cas9 system was used by Shi, et al. [47] to promote the knock-in the 5'-UTR of the *ARGOS8* gene with efficiency of up to 98%. They obtained *ARGOS8* promoter variants, which resulted in improved grain yield under drought stress. Aiming to study heritability of CRISPR mutagenesis, Zhu, et al. [48] edited ZmPSY1 gene that encodes a phytoene synthase (PSY) enzyme. They reported transgenerational gene editing mutations and no off-target effects were detected.

In a different approach, Zong, et al. [49] applied CRISPR base editing strategy in maize, wheat, and rice crops. This approach merges a specific Cas endonuclease (nCas9 or dCas9), a codon-optimized rat cytidine deaminase APOBEC1, and a uracil glycosylase inhibitor (UGI) (**Figure 4**). Researchers edited the CDS of blue fluorescent protein (BFP) to produce the green fluorescent protein (GFP) by shifting one amino acid (histidine to tyrosine) through a **C → T** base substitution, proving to be a suitable strategy to confirm the base editing due to ease of phenotype verification. Recently, Gao, et al. [50] developed an interesting CRISPR approach based on a knock-in strategy in maize for the generation of a complex trait locus (CTL), enabling an easier trait/gene stacking. The authors conclude by arguing that CTL technology may significantly help the development of maize hybrids with multiple transgene-derived traits.

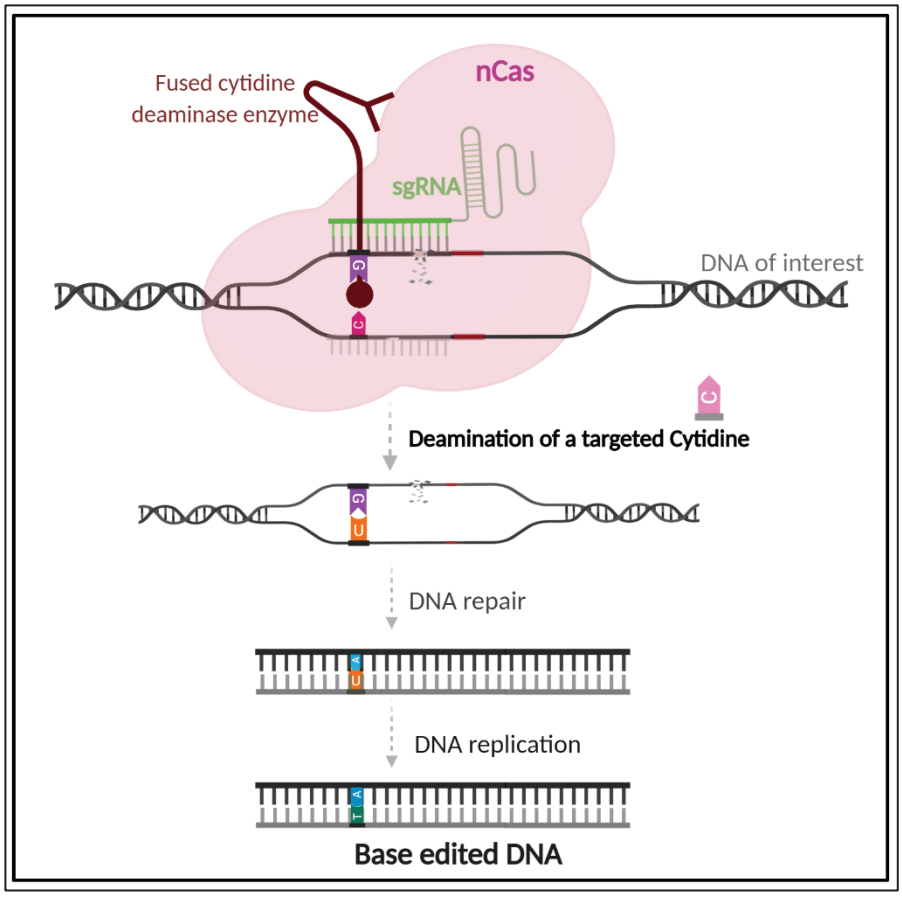

Figure 4. Representation of CRISPR first-generation base editing system mechanism. In this strategy, a catalytically nickase Cas9 endonuclease (nCas9) merged to a catalytic cytidine deaminase enzyme domain is directed by a sgRNA to a specific region in the target genome to create a single-base substitution, converting C:G to T:A, with potential applications in crop trait improvements and correction of single nucleotide polymorphism diseases in farm animals.

5. Genome editing in wheat

Common wheat (*Triticum aestivum* L.) species widely cultivated in the world, representing approximately 20% of the calories in human diet [51]. Wheat is an allohexaploid (AABBDD, 2n = 6x = 42) and due to such a complicated genome, CRISPR/Cas seems to be the most ideal and competent system to improve wheat yield as well as other important agronomical traits, such as resistance/tolerance against biotic and abiotic factors, respectively.

The first work employing CRISPR/Cas system in wheat was performed by Shan, et al. [52] and promote the knock-out of TaMLO gene (Mildew Locus O), which resulted in mutation frequencies of 26.5% to 38%. This study also performed the first CRISPR gene editing in rice, demonstrating the broad applicability of the technique in major crops. CRISPR/Cas system has been applied in wheat using a diversity of approaches and mechanisms with a wide range of aims (table 4). As a rule, all three sets of homologous genes have been targeted at once within A, B, and D respective genomes [53, 54].

Several wheat explants have been used for CRISPR experiments, although protoplasts and immature embryos are predominant. Concerning the transfection method, biolistics, PEG- and *Agrobacterium*-mediated protocols are always used, with only one exception [55]. Bhowmik, et al. [55] performed the CRISPR/Cas9-mediated knock-out of exogenous (*DsRed*) and endogenous (*TaLox2* and *TaUbiL1*) genes from wheat based on the electroporation of microspores (immature pollen) using plasmids containing Cas/gRNA genes, aiming to develop a haploid mutagenesis system. Moreover, they developed WheatCRISPR, an online bioinformatics tool for designing gRNAs for wheat as the host genome.

Gil-Humanes, et al. [56] used an engineered and deconstructed version of the wheat dwarf virus (WDV) genome as a replicon-based system for delivery of CRISPR/Cas9 in immature scutella. The replicon was in a ΔΔ form, in which both movement protein (MP) and coat protein (CP) were deleted in order to allow the increase of heterologous genetic load. Target genes *TaUbi, MLO,* and *EpSps* went through a GFP knock-in with a high frequency of precise gene targeting.

CRISPR base editing was another strategy applied to wheat. Li, et al. [57] used both PEG-mediated transformation of protoplasts and particle bombardment of immature embryos for the delivery of base editing fusion plasmids in order to obtain site-specific base changes (A → G) in wheat and rice.

Concerning the delivery system, Liang, et al. [58] compared CRISPR DNA plasmids with RNP complex and revealed similarities on target mutation efficiencies, whereas off-target activity was at least five-fold lower for RNPs. In another study, Liang, et al. [59] compared RNPs and IVT CRISPR reagents (made up of Cas9 mRNA and sgRNA transcripts) aiming at the optimization of CRISPR plasmid-free delivery protocols, verifying effectiveness and suitability of both systems, besides eliminating plasmid random integration into the host genome. Zhang, et al. [18] compared IVTs and Transiently Expressing CRISPR/Cas9 DNA (TECCDNA) delivery forms. They reported high efficiency of both methods in transiently express CRISPR reagents in bread wheat. Focused on optimizing plasmid-based CRISPR/Cas9 transfection systems, Zhang, et al. [60] demonstrated that *Agrobacterium*-mediated transfection yield a much higher targeting efficiency (54.17%) in wheat immature embryos when compared to PEG-mediated transfection on protoplasts (up to 6.8%).

Strategies to achieve multiplex genome editing in wheat were also performed. Wang, et al. [61] used an approach for multiplex genome engineering to target three genes (*TaGW2, MLO* and *Ta-Lpx1*) responsible for different agronomical traits. In the case of *TaGW2*, mutagenesis in all three homeologous (A, B, D) resulted in a robust increase of grain productivity traits. Addressing both grain quality and public health matters, Sánchez-León, et al. [62] developed a

low-gluten transgene-free edited wheat variety by knocking out α-gliadin genes. The promising results can positively impact human health since gluten proteins from cereals may trigger intense immune responses in patients with celiac disease.

Most recently, Lin, et al. [63] successfully performed the transition of innovative prime editing strategy, so far established only for mammalian cells, to plant systems. Hence, the authors used wheat protoplasts and optimized plant prime editors (PPEs) based on three different reverse transcriptases (engineered M-MLV, CaMV and retron-derived *E. coli* BL21 RT). Prime editing six wheat genes (*TaUbi10, TaGW2, TaGASR7, DME1, TaLox2 and MLO*), they reported events showing INDEL (insertion and deletion) frequencies of 0.5% to 4.9%.

6. Challenges in soybean trait improvement

Soybean (*Glycine max* L.) is a key part of agribusiness, being one of the most profitable agricultural products, and also the major source of oil and protein used in human diet and livestock feed [64]. Native from East Asia, soy has undergone, over the last decades, an expansion greater than any other global crop and its fastest growth occurred in South America [65]. Furthermore, according to FAO, 2017, a high increase in soybean production is expected by 2050.

Due to the great importance of soybean to agribusiness worldwide, genome-engineering studies in this crop have provided valuable insights for discovering new desirable traits [66]. However, the complexity of the soybean genome represents a huge constraint for most mutagenesis approaches. During its evolution lifespan, the genetic material of soybean became a complex paleotetraploid genome after going through one polyploidization and one diploidization. Hence, approximately 75% of soybean genome contains multiple copies of genes, which leads to a high genetic redundancy [67]. In addition, its low transformation efficiency, compared with other model plant species, results in limited forward genetic studies and data availability.

Notwithstanding, such challenges can be addressed, partially, by the application of CRISPR/Cas genome editing technology [68]. To date, CRISPR/Cas genome editing of soybean has shown promising results regarding improvements in agronomic traits that commercially sought-after (table 5). Since much of global soy production is directed to human consumption, the nutrient quality of soybean is an excellent target trait. So far, the distasteful beany flavor of soy, a feature that restricts human consumption, is known to be induced mainly by three lipoxygenase (*LOXs*) genes –*GmLOX1*, *GmLOX2*, and *GmLOX3* [69]. In 2019, aiming at the improvement of soy flavor, Wang, et al. [70] used the CRISPR/Cas9 multiplex system to generate lipoxygenase-free soybean. They demonstrated by colorimetric assay that double and triple CRISPR mutants lost the corresponding LOX activities, paving the way for tastier soybean products.

Table 4. Characterization of CRISPR/Cas strategies used for improvement of wheat (*Triticum aestivum* L.)

Commodity	CRISPR nuclease	Delivery system	CRISPR mechanism	Transfection method	Aim	Explant/ Cell type	Target	Targeting efficiency	Reference
Wheat (*Triticum aestivum* L.)	Cas9	Plasmid	Knock-out	Electroporation	Development of a haploid mutagenesis system for wheat	Microspores	*DsRed TaLox2 TaUbiL1*	92% (*DsRed*)	[55]
Wheat (*Triticum aestivum* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Generation of male sterile lines for hybrid seed production	Immature embryo	*M1*	5%	[53]
Wheat (*Triticum aestivum* L.)	Cas9	Plasmid	Knock-out	Biolistics	Generation of a low-gluten edited wheat	Immature scutella	α-gliadin genes	No data	[62]
Wheat (*Triticum aestivum* L.)	Cas9	Plasmid	Knock-out Knock-in	PEG Biolistics	Targeting of homeoalleles for disease resistance	Protoplast Immature embryo	*MLO*	5.6%	[112]
Wheat (*Triticum aestivum* L.)	Cas9	Plasmid	Knock-out	PEG	Multiplex genome engineering of different agronomic traits	Protoplast	*TaGW2 MLO Ta-Lpx1*	No data	[61]

PEG: polyethylene glycol; MLO: *Mildew Locus O*.

The generation of healthier soybean oil with high oleic acid content could positively affect human health, minimizing lifestyle diseases [71]. In this context, al Amin, et al. [72] used the CRISPR/Cas9 nuclease system to disrupt distinct sites of *FAD2-2 loci* (double identical copies of the genes), responsible for fatty acid profile in soybean. These authors reported mutant seeds with an increased profile of monounsaturated fatty acid, and results showed increased levels (up to 65%) of oleic acid. Aiming to increase soybean productivity by improving plant architecture, Bao et al. [73] employed the CRISPR/Cas9 system to edit *GmSPL9a-d* genes, encoding *squamosa promoter binding protein-like* (SPL) transcriptional factors, all of them usually regulated through *miR156* with negative impacts on soybean plant architecture and yields. Edited plants displaying different loss-of-function mutations showed an increased total node number per plant, reflected in substantial yield improvement.

CRISPR/Cas technology has showed its high potential as a functional genomic strategy and a breeding tool for soybean trait improvement. Hence, to address major critical concerns such as the environmental impact of huge deforestation areas needed for soy cultivation, as well as global food security challenges, the improvement of soybean yield is of imperative importance.

Table 5. Characterization of CRISPR/Cas strategies used for improvement of soybean (*Glycine max* L.).

Commodity	CRISPR nuclease	Delivery system	CRISPR mechanism	Transfection method	Aim	Explant/ Cell type	Target	Targeting efficiency	Reference
Soybean (*Glycine max* L.)	Cas9	Plasmid	Multiplex Knock-out	*Agrobacterium*	Engineer plant architecture to improve yield	Foliar cotyledon	*GmSPL9a* *GmSPL9b* *GmSPL9c* *GmSPL9d*	No data	[73]
Soybean (*Glycine max* L.)	Cas9	Plasmid	Multiplex Knock-out	*Agrobacterium*	Improvement of eating quality	Foliar cotyledon	*GmLox 1* *GmLox 2* *GmLox 3*	No data	[70]
Soybean (*Glycine max* L.)	Cas9	Plasmid	Knock-out	*Agrobacterium*	Improvement of quality	Foliar cotyledon	*GmFad2-2*	21%	[72]

7. Gene editing towards milk quality Improvement

Milk has been a target product for genetic engineering since the 70's, and the efforts towards its improvement have focused on producing milk free from allergens for human consumption, humanization of animal milk and production of milk with high levels of proteins to promote the welfare of neonates or supply a nutritional demand [6]. In this way, CRISPR/Cas gene editing tool emerges as a potential approach to improve animal milk (table 6).

Aiming to decrease the production of β-lactoglobulin (*BLG*), an allergenic compound present in milk, Zhou, et al. [17] generated *BLG* knock-out goats delivering CRISPR reagents through the cytoplasmic injection method. The molecular characterization of edited animals during the lactation period presented a decreased expression in targeted gene and absence of the β-lactoglobulin protein in the milk. This study provided an important caprine model for *β-lactoglobulin*-free milk, an important nutrient source for allergic people, also paves the way for the improvement of cow's milk.

In the same year, Ma, et al. [74] successfully generated a sheep able to produce melatonin (N-acetyl-5-methoxytryptamine)-enriched milk. A down regulation of this important hormonal regulator and potent antioxidant is argued to be associated with metabolic disturbances and neurodegenerative diseases [75].

Table 6. Characterization of CRISPR/Cas strategies used for improvement of milk.

Commodity	CRISPR nuclease	Delivery system	CRISPR mechanism	Transfection method	Aim	Explant/ Cell type	Target	Targeting efficiency	Reference
Milk Goats (*Capra hircus*)	Cas9	Plasmid IVT	Knock-out	Cytoplasmic injection	Generation of *β-lactoglobulin*-free goat milk	Zygote	*BLG locus*	25%-28.6%	[17]
Milk Sheeps (*Ovis aries*)	Cas9	Plasmid IVT	Knock-in	Pronucleous injection	Generation of sheep able to produce milk enriched with recombinant protein melatonin (generation of mammary gland bioreactor)	Embryos	AANAT ASMT	34.69%	[74]
Milk/Meat Cattle (*Bos Taurus*)	Cas9 nickase	Plasmid	Knock-in	Electroporation	Reduction in off target effects	BFF, SCNT	*NRAMP1*	4.5%-5.3%	[76]
Milk/Meat Cattle (*Bos Taurus*)	Cas9	Plasmid	Single base substitution	Electroporation	Repair IARS mutation in Japanese Black Cattle	BFF, SCNT	*IARS*	4.4%	[87]

IVT: *in vitro* transcript; SCNT: somatic cell nuclear transfer; BFF: Bovine fetal fibroblasts.

The authors combined the CRISPR/Cas9 system with constructed marker-free mammary gland-specific expression vectors of AANAT (Arylalkylamine N-acetyltransferase) and ASMT (acetylserotonin methyltransferase), crucial enzymes for melatonin synthesis, and β-casein promoter respectively. The cytoplasm of pronuclear embryos was co-injected with Cas9 mRNA and sgRNA generated by *in vitro* transcription (IVT) together with linearized expression vectors. This approach enabled effective expression of melatonin synthetic enzymes in mammary epithelial cell culture and in edited animals.

The work presented high performance models for melatonin source with several applications in nutrition and medicine, such as the equilibration of melatonin levels in humans and animals. Moreover, the results reveled an efficient gene editing approach for farm animals aiming to yield higher levels of natural melatonin or other interesting molecules in milk.

Although promising, genome editing in animals must face relevant concerns, such as on-target precision of the adopted strategy, strictly related with off-targets undesirable effects. In this context, Gao, et al. [76] employed a catalytically inactive Cas endonuclease (nCas9) to induce a precise gene targeting (knock-in) at a specific locus in cattle´s genome. Using chromatin immunoprecipitation sequencing (ChIP-seq) to identify major binding sites for the Cas9 protein in bovine fetal fibroblast cells (BFFs), the authors demonstrated that nCas9-induced single-strand break (SSB) is able to not only stimulate homology-directed repair (HDR), promoting insertion of the target gene, but also diminish off-target effects *in vitro* (on BFFs) and *in vivo* (on cattle).

These studies open doors for advances in genome editing in domestic animals, raise possibilities for improvements in milk nutritional quality, and to enhance the production of several molecules with biomedical interest. Furthermore, although challenging, these studies show the viability genome editing tools in large and complex livestock species.

8. CRISPR status in livestock meat

Currently, domestic animals, such as pigs, chickens and cattle, represent the most common meat sources in human diet [2, 3]. Myostatin (*MSTN*) gene, a member of the transforming growth factor beta (TGF-B) superfamily, is the mostly common target for improvement of meat yield in domestic animals, and is the most commonly target to CRISPR/Cas knock-out (table 7). It has been reported that the inhibition of MSTN is related to increased muscle and body mass, leading to an increased amount of meat in animals [77, 78].

Table 7. Characterization of CRISPR/Cas strategies used for improvement of meat.

Commodity	CRISPR nuclease	Delivery system	CRISPR mechanism	Transfection method	Aim	Explant/ Cell type	Target	Targeting efficiency	Reference
Meat Goats (*Capra hircus*)	Cas9	Plasmid IVT	Base editing	Cytoplasmic microinjection	Introduce the point mutation in goat genome and improve litter size	Oocyte	*GDF9*	24%	[115]
Meat Goats (*Capra hircus*)	Cas9	Plasmid IVT	Knock-out	Cytoplasmic injection	Generation of double-muscled phenotype in goats	Zygote	*MSTN*	85.7%	[117]
Meat Sheeps (*Ovis aries*)	Cas9	Plasmid IVT	Multiplex Knock-out	Cytoplasmic injection	Multiplex knock-out to improvement of traits with economic importance	Zygote	*MSTN* *ASIP* *BCO2*	27.8% 33.3% 27.8% 5.6% (multiple)	[119]
Meat Pig (*Sus scrofa domesticus*)	Cas9	Plasmid	Knock-out	Electroporation	Introduce two mutations (PVD20H and GP19del) in the SP of *MSTN* in pig breed, Liang Guang Small Spotted to improve meat production	LPK SCNT	MSTN signal peptide	37.2%	[84]
Meat Chicken (*Gallus gallus domesticus*)	Cas9	Plasmid	Knock-out	Lipofection	Reduction of abdominal fat deposition and alteration on Fatty acid composition in chicken	PGC Transplantation	*GOS2*	85.4%	[85]
Meat Chicken (*Gallus gallus domesticus*)	Cas9-D10A nickase	Plasmid	Knock-out	Lipofection	Improvement in meat chicken production	PGC Transplantation	*MSTN*	13.5% (In F1 Generation)	[86]
Meat Chicken (*Gallus gallus domesticus*)	Cas9-D10A nickase	Plasmid	Knock-out	Lipofection	Improvement of chicken economic traits	chicken DF1 cells (*in vitro*)	*MSTN*	100%	[120]

IVT: *in vitro* transcript; SCNT: somatic cell nuclear transfer; LPK: Liang Guang Small Spotted pig kidney cells; PGC: Primordial Germ Cells.

Zheng, et al. [79] proposed a different target and CRISPR strategy to improve meat production. These authors performed the CRISPR knock-in of *UCP1* gene from mouse into a pig´s genome. The target gene is related with brown adipose tissue formation, involved with thermogenesis and homeostasis regulation. Researchers employed a CRISPR/Cas9 knock in-HDR-independent method, to obtain an efficient insertion of mouse adiponectin-UCP1 fragment (9 kb) into the porcine endogenous UCP1 nonfunctional locus. Pig embryonic fibroblast (PEF) cells were co-transfected by nucleofection with plasmids containing Cas9-gRNA, followed by somatic cell nuclear transfer (SCNT). Interestingly, molecular characterization results revealed mismatches at both 5′ and 3′ ends of insertion sites, indicating that this strategy might not fit for precise gene targeting. However, phenotype analysis of animal events showed an improved ability to regulate body temperature reduced fat deposition, and an enhancement of carcass weight, relevant traits regarding meat production.

In the following year, Li, et al. [80] used CRISPR system to promote the knock-in of *fat-1* gene in pigs. Coding for a fatty acid desaturase, fat-1 gene acts on the conversion of n-6 polyunsaturated fatty acids (N-6PUFAS) into n-3 polyunsaturated fatty acids (N-3PUFAS), which play key roles in regulating diverse biological processes.

Additionally, reduced ratios of n-6PUFAs/n-3PUFAs contribute to a healthier human diet [81]. A non-functional enzyme capable of converting n-6PUFAs into n-3PUFAs leads to the production of an animal meat considered unhealthy for human feeding. CRISPR/Cas system was used to insert *fat-1* gene into the porcine Rosa 26 locus of Porcine fetal fibroblasts (PFFs). sgRNA and fat-1-KI plasmid were transfected into PFF cells by electroporation, and *fat-1*-KI-positive PFF cells were used to perform SCNT. Several tissues of F1 generation animals expressing the knock-in gene were detected by gene expression analysis. As transgenic pigs presented a significant decrease in the n-6PUFAs/n-3PUFAs ratio, a meat with an improved nutritional property was successfully achieved.

Using a different approach, Xiang, et al. [82] employed CRISPR/Cas base editing strategy in Bama pigs. The authors aimed to substitute a guanine to an adenine in a known conserved motif (5′-GCTCGC-3′) to prevent the ligation of ZBED6 (BED-type containing 6), a repressor of IGF2-intron 3 (an important locus involved in the increase of lean meat production in pigs). To promote site-specific base editing, they transfected pig embryos with a nickase nCas9 mRNA and a dual sgRNAs targeting SNP sites in *IGF2*-intron 3. Results showed that the base editing of *IGF2*-intron 3 improved meat production. Significant economic traits (i.e., body mass and carcass weight) were increased without disturbing any other general trait present in Bama pigs. The authors indicated the possibility of using the same approach in other Chinese pigs.

Zou, et al. [83] validated the regulatory function of the *Fbxo40* gene as a potential target to improve meat production in pigs. This gene is an important component of *SCF-E3* ubiquitin ligase complex and is responsible for the ubiquitination of IRS1 (insulin receptor substrate 1), inactivating insulin-like growth factor-1 - IGF1/AKT pathway involved in muscle hypertrophy. CRISPR/Cas was used for the generation of *Fbxo40* knock-out animals. Expression vector of Cas9-D10A nickase (Cas9n) and sgRNAs were inserted in PFFs by nucleofection, and positive single-cell colonies were further used for SCNT. Knock-out animals showed desired muscle cells phenotype with increased levels of IRS1, and IGF1/Akt pathway was also activated. Knock-out animals showed no abnormalities and pathological effects were not detected. Moreover, the edited animals produced 4% more muscle mass in comparison to wild type controls. In a recent study, Li, et al. [84] proposed a mutation insertion in PVD20H and GP19del in the signal peptide (SP) of *MSTN* gene of a Chinese pig breed (Liang Guang Small Spotted pig) using the CRISPR/Cas system to knock-out a specific site in the SP. The mutation of PVD20H promoted an increase in muscle mass and MSTN production was not affected. The mutation in the SP of *MSTN* resulted in the activation of PI3K/Akt pathway and lead to an increase in muscle mass by hyperplasia. These results suggested that signal peptide edition can improve muscle development without affecting MSTN peptide production, thus benefiting edited animals.

Besides research with pig species, several studies have also focused on the improvement of chicken meat production using the CRISPR/Cas system. Targeting the decrease of excessive fat deposition in broiler chickens, Park, et al. [85] investigated the biological function of G0S2 gene in chicken fat deposition and lipid metabolic pathways. CRISPR/Cas system was used to generate G0/G1 switch gene 2 (G0S2) knockouts in chicken. G0S2 gene is involved with negative regulation of PNPLA2 (patatin-like phospholipase domain-containing protein 2), an enzyme involved in adipose triglyceride (TG) hydrolysis. In this work, the primordial germ cell line (PGC) was transfected with Cas9-green fluorescent protein co-expression plasmid and multi-guide RNAs targeting the first exon of G0S2 gene. The dorsal aorta of recipient embryos was microinjected with edited cells. Knocked-out animals were evaluated and results showed a relevant decrease in abdominal fat deposition without disturbing any important trait. This study provided important data to a better understanding of the G0S2 role in cell fat deposition, as well as for the generation of genetically improved chickens employing the CRISPR/Cas technology.

Recently, aiming at the improvement of chicken meat production, Kim, et al. [86] employed CRISPR/Cas system to promote knock-out of *MSTN* in chicken. A D10ACas9 nickase was used in the study as a strategy to reduce potential off-targets. They delivered a D10ACas9-GFP co-

expression plasmid and two gRNAs into a chicken's primordial germ cells (PGC) by lipofection, and positive PGCs were then transplanted into the dorsal aorta of recipient embryos. Researchers successfully obtained chicken *MSTN* knockouts displaying a significant increase in breast and leg muscle mass, besides an interestingly decreased abdominal fat deposition. The authors also called attention to the fact that skeletal muscle hypertrophy and hyperplasia presented by *MSTN* knock-out animals can be sex- and muscle type-specific.

Regarding CRISPR gene editing in cattle, there are important studies targeting disease resistance. Gao, et al. [76] used a single nCas9 for gene insertion in a specific locus in the cattle genome. Edited cattle showing enhanced resistance against tuberculosis was obtained with reduced off-target events. Aiming to correct the IARS mutation, Ikeda, et al. [87] used CRISPR/Cas system to repair the genome of Japanese Black cattle. The isoleucyl-tRNA synthetase (IARS) syndrome is caused by IARS mutation, a recessive disease that can cause and problems in intrauterine development and death in prenatal and neonates. Associating CRISPR/Cas with SNTC, authors succeeded in restore normal condition in syndromic animals by substitution of a mutated nucleotide in *IARS* gene. The generation of disease-resistant animals has an important impact on animal welfare and, hence, indirectly represents a relevant improvement in meat production.

These studies established important approaches in the improvement of cattle meat production by using the CRISPR/Cas gene editing tool.

9. CRISPR fishery products

Aquaculture comprises an important activity for food production worldwide, and in recent years it has become the major source of seafood destined for the human diet [3]. In this context, CRISPR/Cas genome editing system is considered as an important tool to accelerate genetic improvements in aquaculture breeding and production [88]. Several studies have been performed in fishes using CRISPR/Cas system (table 8).

In this context, optimization of muscles without intramuscular bones is highly desirable and presents a challenge in production of common carp, species with high economic importance. Employing CRISPR/Cas and TALEN editing tools, Zhong, et al. [89] disrupted 4 genes (*SP7*, *RUNX2, SPP1*, and *MSTN*) by TALEN and 2 genes (*SP7A* and *SP7B*) by CRISPR/Cas in the genome of common carp (*Cyprnus carpio*). All transgenic carp presented improper formation of bones and TALEN *MSTN* knock-out events showed increase in muscle mass.

Aiming to improve catfish (*Ictalurus punctatus*) production, Khalil, et al. [90] used CRISPR/Cas system to disrupt MSTN gene. Cas9 enzyme and three gRNAs (targeting different exons) were applied individually and mixed into catfish zygote embryos by microinjection. Results demonstrated elevated mutation efficiency (88% - 100%) and enhanced survival of embryo hatch (42%) and early fry stages (90%), with no anomalies detected in edited animals. Regarding important traits, authors reported an increase in hyperplasia (33.7%) and hypertrophy (2%) of muscle fibers, and also an increase of 29.7% in body weight, when compared to wild type animals. This study was the first to use a purified Cas9 protein, hence, representing an interesting strategy to increase editing efficiency on aquaculture species.

Kishimoto, et al. [91] developed a new breed of *Pagrus major* (red sea bream), an important commercial species in aquaculture production, using CRISPR/Cas system to knock-out *MSTN* gene. Delivering Cas9 mRNA and three sgRNAs by microinjection into the cytoplasm of fertilized eggs, they reported high rate of INDEL mutations (80%-100%) on the first exon of *MSTN* gene in G0 individuals.

Table 8. Characterization of CRISPR/Cas strategies used for improvement of aquiculture

Commodity	CRISPR nuclease	Delivery system	CRISPR mechanism	Transfection method	Aim	Explant/ Cell type	Target	Targeting efficiency	Reference
Aquiculture products: Common Carp (*Cyprinus carpio*)	Cas9	IVT	Multiplex Knock-out	Microinjection	Optimization of the muscle production without bones intramuscular	Embryo	*MSTNba SP7A*	60.1% 63.4% (90% double)	[89]
Aquiculture products medaka (*Oryzias latipes*)	Cas9	Plasmid	Knock-out	Microinjection	Evaluation of role of *MSTN* gene in medaka growth	Embryo	*MSTN*	No data	[121]
Aquiculture products Catfish (*Ictalurus punctatus*)	Cas9	IVT	Knock-out	Microinjection	Improvement of catfish muscle production	Embryo	*MSTN*	88% - 100%	[90]
Aquiculture products Red sea bream (*Pagrus major*)	Cas9	IVT	Knock-out	Cytoplasmic injection	Establishment of a fish breed with enhanced muscle production	Fertilized eggs	*MSTN*	100%	[91]

IVT: *in vitro* transcript.

After two years, a new breed (*MSTN*) was obtained and edited fish presented an increase of 16% in skeletal muscle with a moderate increase in body width and height. Off-target mutations were not detected.

Therefore, all mentioned groundbreaking CRISPR/Cas studies performed in major plant crops, livestock, and fishery, supported by the recent advances in CRISPR genome editing, including enhancements in the efficiency of CRISPR knock-in/knock-out strategies, delivering of CRISPR reagents, and DNA-free constructs, have generated knowledge and evidence for improvement of important productive/quality-related traits in different organisms, playing a crucial role for the development of a sustainable agribusiness worldwide.

10. Prospects on the horizon

Modern agribusiness represents a fruitful and long-lasting union between scientific research and technology that has been leading to unprecedented improvements in various food products consumed by humans. Agriculture, livestock, and aquaculture commodities represented mainly by plant crops, farm animals, and fish, respectively, are the natural resources responsible for feeding the world. Even though remarkable advancements have been achieved in plant and animal conventional breeding in the last few decades, the global rising call for food poses a huge challenge for sustainability in agribusiness. CRISPR/Cas9 gene-editing technology has revolutionized bioscience and biotechnology in the way of performing trait-improvements in plant crops and animals due to its high precision, versatility, and relative ease of use. In addition, as the CRISPR toolbox widens at an unprecedented pace, impressive researches have been carried out, and its concrete application in agribusiness is already on the horizon.

Potential innovation in agribusiness products through the use of CRISPR/Cas technology has been hindered by inefficient and costly methods of transformation and regeneration of edited cell targets. In plants a real bottleneck that should be addressed is the cycle of transformation that is highly dependent on the delivery of CRISPR compounds mediated by *Agrobacterium* and also on the time-consuming, and labor-intensive traditional tissue culture to regenerate viable edited plants. Despite being the first-choice method for CRISPR plant transformation, it may cause unintended changes in plant genome and epigenome. Additionally, many crops are recalcitrant to regeneration, which limits the use of CRISPR technology [92].

Therefore, novel and improved existing delivery systems are emerging as a key to overcome barriers and to broaden CRISPR gene editing in plants. Recently, Maher, et al. [93] reported an innovative method to generate CRISPR edited plants. In this study, developmental regulators and CRISPR reagents were delivered to somatic cells of a whole plant and, through *de novo* meristem induction, DNA modifications were passed to the next generations, bypassing the need for tissue culture. Well established in animal models, DNA-free genome edited murines are routinely produced by delivering *IVT* Cas9 mRNA and sgRNA into zygotes (one-celled embryos) through direct injection [6]. Such strategy is highly desirable for application in crop plants because it addresses relevant GMO legislative concerns.

The delivery of preassembled RNP complex to obtain DNA-free genome editing has been performed in crop plants such as rice protoplasts derived from somatic foliar tissue or maize and wheat embryo cells with transfection mediated by polyethylene glycol (PEG) and biolistic (gene gun) particle bombardment, respectively [42, 59]. However, isolation, culture, differentiation, and regeneration of protoplast-derived edited plants is labor-intensive, and still not feasible for most plant crops. Besides, biolistics particle delivery causes major damage in the targeted tissue, and the frequency of rescuing viable events is extremely low [94]. Recently, Toda, et al. [95] described a new and highly efficient CRISPR DNA-free genome editing system in a rice model crop. For the first time, RNP complex was delivered directly into rice zygotes produced by *in vitro* fertilization, producing viable mature edited plants in the absence of selective agents and random integration of transgenes. Besides encompassing major genome engineering concerns, the study also holds great potential to be applied to other important crop species.

The major challenge in genome edition in animals has been the difficulty in applied the technique, based major in HR associated with SCNT. In this way CRISPR/Cas system, has been promoting a new perspective in livestock research, this technology allows direct targeted genome modification in one step by microinjection in zygotes. However, additionally with technical challenges off-target effects, ethics and generation of genetically modified organisms also are current challenges in gene edition of farm animals.

A precise gene insertion or replacement in a genome holds a tremendous potential to reshape agribusiness worldwide through the introduction of new trait-carrying crop varieties ideally adapted to modern agricultural practices and aspirations or even correcting/replacing undesirable traits in farm animals. However, mastering its use still needs some troubleshooting, and CRISPR/Cas gene-editing technology seems to have the right toolbox to do it. In general, CRISPR/Cas system consists of a two-stage process comprising (1) generation of single- or double-stranded breaks (SSB/DSB) in a specific DNA target site, or nucleotide deamination (for CRISPR base editing) and (2) induction of different host genome repair routes to fix the damaged sites [96]. In most plant cells, DNA damage is repaired, basically, by two major pathways known as non-homologous end joining (NHEJ) and homology-directed repair (HDR), the latter being imperative to the success of HDR-mediated CRISPR/Cas GT strategy as it relies on the replacement/insertion of an exogenous donor template stretch of DNA [97]. However, somatic cells of higher plants mostly employ NHEJ to repair DNA damage (at least two orders of magnitude more frequently than HDR), which represented up to now one of the major obstacles for the effective application of such a powerful technique [98]. Notwithstanding, Anzalone, et al. [99] recently reported a novel CRISPR/Cas gene targeting approach called 'prime editing', which is based on a nickase Cas9 (H840A) fused with a reverse transcriptase enzyme (RT), which does not require DSBs or a donor template for the addition of DNA modifications to specific target sites. The novel strategy represents a milestone in the CRISPR gene targeting approach and shows high potential to revolutionize trait improvement in agribusiness bioproducts.

Modern plant cultivars and animal breeds evolved from artificial selection (domestication through intensive inbreeding cycles), where introgressed characteristics favored production. On the other hand, artificial selection resulted in narrow genetic diversity that has led to increased

susceptibility to biotic and abiotic stresses, resulting in negative impacts on plant crop and animal productivity. In this context, CRISPR/Cas technology stands as an efficient and precise tool with great potential for improving plant and animal design and *de novo* domestication of elite cultivars and breeds, aiming to expand genetic diversity and thus ensuring that agriculture, livestock, and aquaculture remain sustainable worldwide.

One of the greatest challenges to be faced with the application of CRISPR technology to agribusiness is not technical, but political and social. However, the scientific community must keep providing complete transparency throughout the publication of accurate results, and public trust and agreement have a key role in the process, influencing regulatory policy approvals. Ethical concerns are frequently associated with genetic engineering and biotechnology approaches. Synthetic biology, considered the most recent strategy of molecular biology to generate GMOs, has also faced the same problems involving society and the scientific community in the last decade, especially when linked to biosafety worries. In this way, biosafety of CRISPR/Cas products mainly regarding off-target mutations have been widely debated, and huge efforts have been applied towards the improvement of software to predict off-targets and design of highly specific guide-RNAs, as well as a novel repertoire of high-fidelity variants and orthologous of Cas9 nuclease. In plants and animals, all those improvements and stringent protocols enabled us to detect any off-target mutation and to remove it from the end product by a breeding technique of hybridization, which leads to a final transgene-free edited bioproducts. This alternative uses Mendelian gene segregation to eliminate the transgene (when using plasmid DNA) while maintaining targeted mutagenesis previously achieved in the presence of expressing Cas nuclease. Besides, transgene-free crops and animal breeds can also be generated by using delivery systems other than plasmids, such as RNPs, IVTs, and viral replicons, which remain episomal in the cytoplasm or in the nucleus, without transgene integration into the target genome. These strategies may be advantageous to bypass biosafety regulations, contributing to the faster transition of basic studies from the bench to applied research in the field. Ultimately, it is conceivable through all the above-mentioned recent advancements on CRISPR genome editing in the agribusiness field that CRISPR bioproducts hold great potential to increase the productivity of important commodities, solve critical agriculture issues worldwide, and improve human life quality, ensuring sustainable global food supply.

Author Contributions

Mariana Rocha Maximiano*, drafted the manuscript and wrote the abstract, introduction and topics related with animal gene editing, prospect on the horizon and designed the figure 1, table 1 and supplementary table (5-7). **Fabiano T. P. Távora***, wrote the abstract, introduction, topics related with rice and soybean gene editing, prospect on the horizon and designed figures (2-4) and supplementary table (1 and 4). **Guilherme Souza Prado** wrote topics related with wheat and maize gene editing, prospect on the horizon and designed supplementary table (2 and 3). **Simoni Campos Dias** supervised the write manuscript. **Angela Mehta** supervised the write manuscript**. Octávio Luiz Franco** drafted the manuscript and supervised the write manuscript. **All authors** provided critical feedback and helped shape the research, analysis and manuscript.

*These authors contributed equally.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter II:

Shotgun proteomics coupled to transient-inducible gene silencing reveal rice susceptibility genes as new sources for blast disease resistance

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Shotgun proteomics coupled to transient-inducible gene silencing reveal rice susceptibility genes as new sources for blast disease resistance

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ABSTRACT

A comparative proteomic analysis between two near-isogenic rice lines, displaying a resistant and susceptible phenotype upon infection with *Magnaporthe oryzae* was performed. We identified and validated factors associated with rice disease susceptibility, representing a flourishing source toward a more resolute rice-blast resistance. Proteome profiles were remarkably different during early infection (12 h post-inoculation), revealing several proteins with increased abundance in the compatible interaction. Potential players of rice susceptibility were selected and gene expression was evaluated by RT-qPCR. Gene Ontology analysis disclosed susceptibility gene-encoded proteins claimed to be involved in fungus sustenance and suppression of plant immunity, such as sucrose synthase 4-like, serpin-ZXA-like, nudix hydrolase15, and DjA2 chaperone protein. Two other candidate genes, picked from a previous transcriptome study, were added into our downstream analysis including pyrabactin resistant-like 5 (*OsPYL5*), and rice ethylene-responsive factor 104 (*OsERF104*). Further, we validated their role in susceptibility by Transient-Induced Gene Silencing (TIGS) using short antisense oligodeoxyribonucleotides that resulted in a remarkable reduction of foliar disease symptoms in the compatible interaction. Therefore, we successfully employed shotgun proteomics and antisense-based gene silencing to prospect and functionally validate rice potential susceptibility factors, which could be further explored to build rice-blast resistance.

Significance: R gene-mediated disease resistance is race-specific and often not durable in the field. More recently, advancements in new breeding techniques (NBTs) have made plant disease susceptibility genes (S-genes) a new target to build a broad spectrum and more durable resistance, hence an alternative source to *R*-genes in breeding programs. We successfully coupled shotgun proteomics and gene silencing tools to prospect and validate new rice-bast susceptibility genes that can be further exploited toward a more resolute blast disease resistance.

1. Introduction

Rice (*Oryza sativa* L.) is among the most important food crops worldwide and stands as a major source of calories for half world's population [1]. However, both biotic and abiotic stresses affect rice growth. Among biotic stresses, infection by *Pyricularia oryzae* (teleomorph *Magnaporthe oryzae*), a hemibiotrophic fungus responsible for rice blast, consists of a major constraint for rice production that leads to a serious threat to food security worldwide. Moreover, according to Priyanka Jain and colleagues [2], it is estimated that the annual global losses caused by this disease would be enough to feed 60 million people. Therefore, it is vital to develop effective means to control this disease to ensure global food security.

To combat invasion by plant pathogens, plants have evolved an array

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of defense systems, from physical/chemical preformed defense barriers to pathogen-molecular patterns/effector-triggered immunity, known as PTI and ETI, respectively [3]. Therefore, all stringent defense barriers imposed by the host have forced the pathogen to coevolve, resulting in a high degree of host specialization that enabled the pathogen to circumvent host pre-formed and pathogen-inducible defenses [4]. Adapted pathogens such as *M. oryzae*, have evolved effector proteins and small molecules to suppress PTI and can thereby establish an Effector-triggered susceptibility (ETS) [5]. Besides, research on the mode of action of those effectors has revealed its capacity of manipulating host target proteins, mostly aimed at disabling the plant immune system [6]. Even more intriguing, plant-derived factors can auto-trigger host susceptibility by serving as signal cues for pathogens attachment, germination, accommodation, and maintenance [7].

To date, the most important methods for blast control are fungicide application, cultural practices, and cultivation of resistant varieties, the last being the most used and environment-friendly approach which, unfortunately, only partially controls blast disease [8]. However, plant natural resistance is generally short-lived when conferred by a single gene or a few major genes [9]. In the last decades, researchers have been striving to breed for resistance against blast disease and rice cultivars harboring several resistance genes were developed [10]. However, along with being highly time-consuming and a labor-intensive technique, conventional breeding of *R* gene-mediated resistance also breaks down within few years after their commercial use due, in part, to the high degree of genetic variability of *M. oryzae* [11].

The well-established concept of the gene-for-gene relationship between host resistance (*R*) and pathogen avirulence (*AVR*) genes [12], drives the common-sense understanding of plant-pathogen incompatible (resistant) or compatible (susceptible) interaction outcomes. Hence, for the majority of plant diseases, the genetics of susceptibility is less tangible. In fact, there is mounting evidence of plant disease susceptibility to being more than a failure of host immunity. Although the plant-pathogen arms race has forced pathogens to continuously evolve new strategies to evade or suppress plant immunity, most pathogens, especially hemibiotrophic fungus, require the cooperation of the host for the establishment of a compatible interaction [4]. Thus, all plant genes that somehow facilitate infection and/or support compatibility can be considered as a susceptibility (—*S*) gene [7]. One of the best-known 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 [13]. Moreover, *Mlo* mutants represent the potential robustness of *S*-gene strategy, of which a recessive mutant 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. Several promising studies already indicate that the rational manipulation of host susceptibility can contribute to the development of effective disease management strategies, making it an interesting alternative approach to *R* genes in breeding programs [14]. Nonetheless, the identification of host genes responsible for triggering disease susceptibility as well as the molecular understanding of how susceptibility operates still remarkably limited.

In this scenario, aiming to reveal and characterize proteins encoded by potential rice *S*-genes, we performed a shotgun proteomics analysis on both susceptible and resistant near-isogenic rice lines (NILs)-*M. oryzae* interactions at the early stages of infection, 12 and 24 h post-inoculation (hpi). Our results revealed potential *S*-gene-encoded proteins, differentially-abundant in the susceptible interaction 12 hpi, that seem to be involved in fungus accommodation, growth/sustenance, and suppression of plant immunity. We further investigated gene function *in planta* of potential candidates by performing TIGS assay, exploiting the delivery of short antisense phosphorothioate-modified oligo-deoxyribonucleotides (PTOs) [15]. This alluring gene silencing approach enabled us to validate a couple of our most prominent targets as functionally engaged with rice-blast susceptibility, revealing a naked eye clear-cut decrease of foliar disease symptoms in the rice-*M. oryzae* compatible interaction, probably due to the negative modulation of corresponding transcripts. This study provided a clearer molecular understanding of the acuities of rice-blast compatible interaction, revealing factors with potential key roles in triggering a host-susceptibility disease state. The development of alternative approaches for genetic improvement of plant resistance holds the potential for innovative breeding technologies, which could circumvent limitations of conventional strategies providing, ultimately, greater blast resistance durability in rice paddies.

2. Materials and methods

2.1. Plant materials, bioassay, and protein extraction

Seeds of rice NILs resistant (IRBL5-M) and susceptible (IRBLi-F5) to *M. oryzae* (isolate 9881) developed by IRRI-Japan Collaborative Research Project were obtained from Embrapa Arroz e Feijão (courtesy of Dr. Raquel Mello). All rice plants were grown in a greenhouse with a temperature of 28 °C, relative humidity of 85%, and a photoperiod of 12 h. The fungus inoculum was prepared as described in Filippi MC & Prabhu AS [16] and the concentration was adjusted at 3×10^5 conidia per ml. Rice NIL plants with three expanded leaves were inoculated with spore suspension by atomized spraying, incubated in a humid chamber for 24 h at 20 °C, and subsequently maintained in the greenhouse at temperatures ranging from 25 to 28 °C. For the analyzes, rice leaves were harvested at 0 (uninfected control), 12, and 24 hpi. Three biological replicates were used in this study. Rice NILs IRBL5-M and IRBLi-F5 were maintained for 7 days for observation and quantification of symptoms as described in Notteghem [17]. The total proteins were extracted with phenol and precipitated with ammonium acetate in methanol, according to Carmo et al. [18]. Protein quantification was performed using Qubit™ Protein assay kit (Thermo Scientific®).

2.2. Protein digestion and sample desalting

Extracted proteins (approximately 10 μg) were solubilized in 60 μL of 50 mM ammonium bicarbonate (pH 8.5). For protein trypsinization, the following steps were performed as described by Murad and Rech [19], with some modifications. Briefly, 25 μL of *Rapi*GEST™ (Waters®, USA) (0.2% *v*/v) was added to the solubilized proteins. Next, the samples were vortexed and incubated in a dry bath at 80 °C for 15 min. After that, 2.5 μL of DTT 100 mM was added and samples vortexed gently, followed by incubation at 60 °C for 30 min. Then, 2.5 μL 300 mM of iodoacetamide solution was added, samples briefly vortexed, and incubated in the dark at room temperature for 30 min. Subsequently, samples were digested with 2 μg of trypsin (Sigma-Aldrich®) at 37 °C in a dry bath overnight. After digestion, 10 μL of a TFA 5% solution was added, the tubes incubated for 90 min at 37 °C in a dry bath, and centrifuged at 14000 *xg* at 6 °C for 30 min. The supernatant was transferred to a new tube and vacuum dried. For protein quantification samples were solubilized in 50 μL of ultra-pure water. The samples were desalted according to Rappsilber et al. [20], with modifications. In brief, to set up the desalting columns, solid-phase C18 discs (EMPORE™ - 3 M®) were used. Next, prepared columns were washed with methanol and centrifuged for 30 s. Further, a 20 μm R2 Reversed-Phase Resin (POROS™ - Thermo Fisher Scientific®) diluted in solvent B (0.1% formic acid/98% acetonitrile was added and the columns centrifuged for 30 s. After, a solvent A (0.1% formic acid/2% acetonitrile) was used to wash twice each column and then centrifuged at the same rotation. Then, samples were solubilized in solvent A, loaded in the settled column, and centrifuged for 2 min. Finally, desalted samples were eluted with 20 μL of solvent B and vacuum dried. Prior injection into the mass spectrometer samples were solubilized with 0.1% formic acid at a final peptide concentration of 0.5 μg.μL-1.

2.3. LC-MS/MS peptide analyses

After tryptic digestion and desalting, peptides were submitted to a liquid chromatography low flow separation (Dionex Ultimate 3000 RSLCnano UPLC, Thermo, USA). A total of 3 μg of peptides was loaded into a trap column (3 cm × 100 μm), containing C18 particles 5 μm for concentration, 120 Å (ReprosilPur, Dr. Maich GmbH). Next, peptides were eluted from the trap column to an analytical column (24 cm × 75 μm), containing C18 particles 3 μm, 120 Å (ReprosilPur, Dr. Maich GmbH). Samples (1 μg of peptides) were applied to the trapping column and then subjected to linear gradient elution between solvents A (formic acid 0.1% in water) and B (formic acid 0.1% in acetonitrile) of 2% B at 35% B for 155 min under a flow rate of 230 nL/min. Then, fractions were eluted directly into the ionization source of an Orbitrap Elite™ mass spectrometer (*Thermo*, USA) and analyzed in DDA mode (data-dependent acquisition). MS1 spectra were generated in the Orbitrap analyzer (with a resolution of 120,000 FWHM at 400 *m/z*) between 300 and 1650 m/z. For each MS1 spectrum, the 20 most intense ions above the 3000-intensity limit were selected and directed to CID (dissociation induced collision) fragmentation with the automatic gain control (AGC) of 1×10^6 and maximum fill time (TI maximum) of 100 ms. The reanalysis of fragmented ions was inhibited by dynamic exclusion, favoring the identification of less abundant peptides.

2.4. Proteomics data processing

The spectra obtained were aligned and the peptides quantified using *Progenesis® QI proteomics* data analysis software (*Nonlinear Dynamics™*). Protein identification was performed using *Peaks®* Studio software (*Bioinformatics Solutions Inc.™*). In brief, the sequences were inferred from fragmentation data and searches performed on Rice Proteome database - OryzaPG-DB (http://oryzapg.iab.keio.ac.jp/). The search parameters were mass tolerance of precursors of 10 ppm, MS/MS tolerance of 0.5 Da, carbamidomethylation (CAM) as fixed modification, methionine oxidation, and acetylation (N-terminal protein) as variable modifications. Up to two missed cleavages were tolerated by trypsin digestion enzyme. Proteins groups/numbers were filtered under 1% false discovery rate (FDR), corresponding to a -10logP value of 23.5 for the whole proteome dataset. Quantitative analysis was performed by chromatogram feature alignment of the extracted ion chromatograms, followed by extracted peak area quantification and median normalization. Identified proteins were submitted to ANOVA test and the significance *p*-value threshold was set to 0.05. Only proteins containing at least two identified peptides were considered for further discussions and experiments, except for one (1) single peptide-based identified protein (Supplementary Table S1 – spreadsheet 2, accession B9FCZ8). Partial Least Squares Discriminant Analysis (PLS-DA) multivariate statistical analysis was performed using MetaboAnalyst software [21] for homogeneity evaluation between conditions and replicates. Finally, protein enrichment analysis (functional annotation) was done by Blast2GO® software 5.1v. All generated data comprising chromatography, mass spectrometry, protein quantification, and statistics, were deposited into the international repository *Center for Computational Mass Spectrometry* (University of California, San Diego) and into the *ProteomeXchange consortium*, under the IDs MSV000082657 and PXD010542, respectively. Upon completion of the proteomic analysis, a specific set of rice proteins potentially involved in *M. oryzae* susceptibility were selected to further analyses of mRNA transcript expression levels by RT-qPCR.

2.5. RNA isolation and qRT-PCR analysis

Total RNA isolation was performed using TRI reagent (Sigma-Aldrich®). The isolated RNA was quantified by a Nanodrop™ spectrophotometer (Invitrogen®) and used for reverse transcription reaction performed with Next Generation III™ M-MLV enzyme kit (DNA Express Biotechnology). Rice cDNA sequences were used for amplification of *S*-candidate genes using specific primers designed using the software Primer 3. qRT-PCR reactions were performed using SYBR™ Green according to Applied Biosystems® detection system. The cDNAs amplification were quantified by relative quantitative expression method. Efficiencies were calculated by the equation: $E = 10(-1/\text{slope}) - 1$. Cycle thresholds (Cts) were automatically obtained by 7300™ Real-time PCR machine (Applied Biosystem®) and relative expression estimated for each gene according to ($2\text{-}\Delta\Delta Ct$) equation, where $\Delta\Delta Ct = Ct$ Target – Ct reference. *S*-candidates expression was normalized to *OsGAPDH* and *OsEF-alfa* reference genes expression. All reactions were done in triplicates.

2.6. Transient-induced gene silencing (TIGS) for functional validation

PTO-based TIGS methodology described in Lambertucci et al. [15] was followed with some modifications. The CDS sequences of our *S*-candidates were retrieved from the Rice Genome Annotation Project website (http://rice.plantbiology.msu.edu/index.shtml). The design of silencing antisense phosphorothioate-modified oligo-deoxyribonucleotides (PTOs) was performed using the web-based software Sfold (available at http://sfold.wadsworth.org/cgi-bin/soligo.pl), with complementary analysis concerning PTO efficiency parameters made on Oligowalk software (available at http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk). 19-mer short antisense oligos presenting the highest probability of causing silencing of our targets were selected (*PTO1*_OsDjA2:TTTCTAACACGAACACGCC;*PTO2*_OsERF104:ACAATGTGTCGGACTTGGC; *PTO3*_OsPYL5:AAGTGCTTGTACGCCTGCG). Next, specificity to the target transcript was analyzed through an alignment against rice genome and transcriptome database (*Oryza sativa* L. spp. Japonica), using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/) and the exact coordinates of PTO hybridization in the targeted transcript was also visualized on Gramene website (https://gramene.org). A random nonsense PTO (*PTO4*_Random: CTTTTCCTATACGCGGCTT) having no complementarity with any transcript sequences in rice RefSeq RNA database, was designed and used as the 'no-target' negative control. The delivery of PTOs into intact leaves of susceptible rice NIL 3-leaf stage was performed via pressure infiltration, as described in Sparkes et al. [22]. and Dalakouras et al. [23], with modifications. Briefly, PTOs were dissolved in sterile distilled water [10 μM] and infiltrated into rice intact leaves (abaxial face) with a syringe without a needle (0.5 ml). At 16 h post-PTO infiltration, treated plants were inoculated with *M. oryzae*, and the severity of plant symptoms was evaluated 7 dpi using the software Quant®, according to Vale, F.X.R. et al. [24]. The one-way ANOVA and the post hoc two-sample *t*-test was performed for significance (p-value ≤ 0.05) of percentage of healthy foliar surface between treatments and controls. Each treatment was performed on three biological replicates with three independent technical replicates per plant ($N = 9$ leaves).

3. Results and discussion

3.1. When proteome of rice - M. oryzae interaction reveals beyond R gene-mediated resistance

To portray the foundation of rice blast susceptibility responses, we have conducted a comparative proteomic study on compatible and incompatible rice-*M. oryzae* interactions at 12 and 24 hpi. Rice NIL IRBL5-M and IRBLi-F5 (developed and better described in Kobayashi et al. [25]) carrying *Pi5* and *Pii* blast-resistance genes, respectively, were used in the study. In our analysis, IRBLi-F5 and IRBL5-M displayed a susceptible and resistant phenotype, respectively, upon infection with *M. oryzae* isolate 9881, as expected (Fig. 1). To understand the differences in protein abundance among both lines in response to *M. oryzae*, we performed a proteomic analysis. Plants with three expanded leaves were sprayed with fungal spore suspension and leaves were harvested at 0 (mock-inoculated with spore-free water), 12, and 24 hpi. LC-MS/MS comparative shotgun proteomics revealed a quite similar total

proteome profile between rice compatible and incompatible interactions with *M. oryzae* (Supplementary Table S1 – spreadsheet 1), reflecting its common genetic background from Lijiangxintanheigu (LHT), a susceptible Japonica-type rice variety used as recurrent parent in the generation process of rice NILs. Notwithstanding, the identification of an extensive number of differentially-abundant proteins (DAPs) (Supplementary Table S1 – spreadsheet 2), whereby major fluctuations were noted at the most critical early stage of infection (12 hpi), with differentially-increased proteins overcounting the decreased ones (Fig. 2), suggests considerable singularities in proteome regulation between susceptible and resistant rice lines. DAPs were selected for further discussion and analysis based on the fold change ≥ 2 and statistical significance of the difference among conditions (ANOVA *p*-value <0.05). The quality of such differences was evidenced by the smaller %CV intra-conditions (among replicates) than the %CV inter-conditions (Supplementary Table S1 – spreadsheet 2), as well as by the multivariate PLS-DA analysis showing closer replicate groups than condition groups (Supplementary Fig. S1 A-B).

After protein identification and quantification analysis, identified regulated proteins were clustered according to their relative abundance profiles (Supplementary Fig. S2 A-B). This representation divides the total set of DAP proteins ($n = 830$) in groups (clusters #1 to #7) according to their relative abundance profile similarity among the six experimental conditions (i.e., susceptible interaction - S0, S12, S24 hpi; resistant interaction - R0, R12, and R24 hpi, respectively represented by the six columns in Fig. S2 -B). Clusters #4 to #7 seemed to concentrate the most prominent differentially regulated proteins between compatible/incompatible interactions at 12 hpi (up-regulated in S12 and down-regulated in R12), the early stage of infection, suggesting the existence of a specific set of increased proteins potentially linked with host susceptibility upon *M. oryzae* infection. To better depict this putative set of candidate proteins, we generated correlation heatmaps (data available upon request) between normalized patterns of protein abundance and clustering conditions at 12 hpi. In Fig. 3, we highlight the proteins showing the highest contrast in abundance between IRBLi-F5 and IRBL5-M and the lowest *p-values* among the 160 DAPs identified at this early stage of infection. This cut-off parameter revealed a group of 25 differential proteins, increased in IRBLi-F5, which may have an important role in susceptibility, some of which will be discussed below.

3.2. Gene ontology annotation discloses molecular responses underlying rice-blast susceptibility

DAPs identified at the early stage of infection in the comparisons S_12 hpi/S_mock-inoculated; R_12 hpi/R_mock-inoculated; and S_12 hpi/R_12 hpi, were functionally analyzed according to their Gene Ontology (GO) annotations (Supplementary Fig. S3 A-B). The GO analysis results showed that the major functional category predicted in the comparison between compatible and incompatible interactions at 12 hpi (S_12 hpi/R_12 hpi) was related to metabolic processes (20% - organic metabolism; 18% - primary metabolism; 17% - cellular metabolism). Out of the 211 DAPs identified in this comparison, 149 were associated with this GO term, ultimately indicating that *M. oryzae* infection of rice susceptible NIL IRBLi-F5 elicits a highly positive and early regulation of host metabolic genes. Next, to highlight over-represented functional categories, an enrichment analysis of GO terms based on a specific reference dataset for *Oryza sativa* was performed using *PHANTER* classification system (available at http://pantherdb.org). These results also revealed that 'Cellular metabolic process' was the biological process most over-represented in the comparison S_12 hpi/R_12 hpi (*p*-value = 2.98^{-5}) (Table 1).

3.3. A SWEET welcome to guests

Although the intricate mechanisms by which *M. oryzae* reprogram host metabolism during plant infection are poorly understood, there is mounting evidence showing that most host-adapted pathogens rely on the activation and/or upregulation of certain plant genes (known as susceptibility *S*-genes) to meet their metabolic (carbon and other nutrients) requirement for proliferation and spread. *M. oryzae* seems to co-opt host metabolism to obtain nutritional advantages [26], highly demanded by its intense host intracellular multiplication, crucial for suppression of defense responses and disease establishment. The most known archetype of plant *S*-gene regarding host primary metabolism

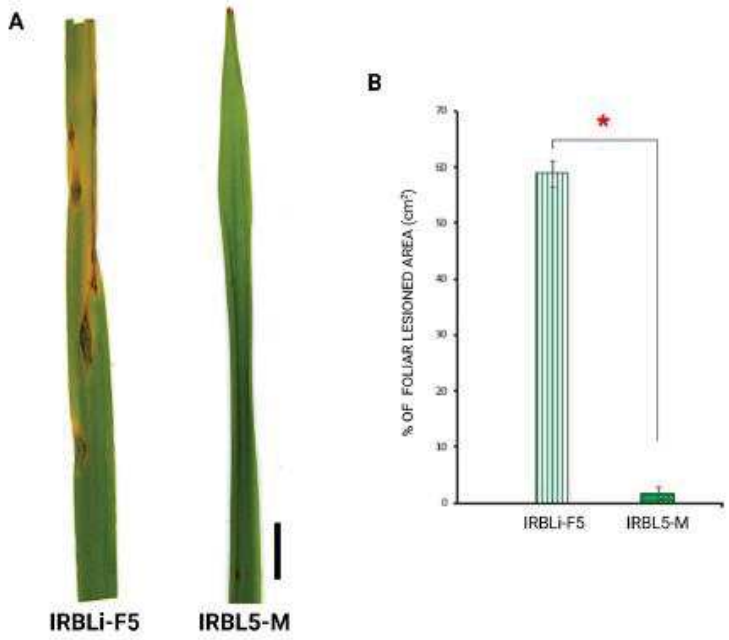

Fig. 1. Phenotype of rice NILs inoculated with *M. oryzae* isolate 9881. (A) Leaf lesions on susceptible (IRBLi-F5) and resistant (IRBL5-M) NILs observed at 7 days post-fungus inoculation (dpi). Scale bar = 1.0 cm. (B) Percentage of the injured leaf area measured at 7 dpi. Asterisk between the means denotes a statistically significant difference (ANOVA, p-value ≤ 0.05). Error bars represent the standard deviation of the mean considering 10 biological replicates in each condition.

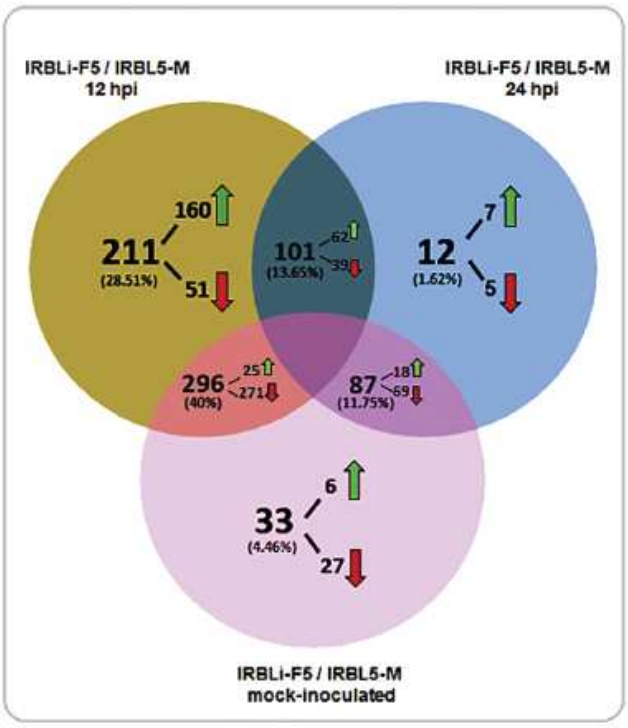

Fig. 2. Venn diagram of differentially-abundant proteins (DAPs) identified from comparative shotgun proteomic analysis in the comparison between IRBLi-F5 and IRBL5-M NILs under the 3 conditions (12, 24 hpi, and mock-inoculated). Increased and decreased proteins are represented by the green and red arrows, respectively. IRBLi-F5 and IRBL5-M (rice NILs susceptible and resistant to *M. oryzae* isolate 9881, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

consists of the *SWEET* (Sugars Will Eventually be Exported Transporters) gene family. Even though sucrose, the final product of photosynthesis, is the primary sugar transported in the phloem of most plants, pathogens prefer reduced forms of carbohydrates, such as hexose monomers, i.e.: glucose and fructose. Thus, to have access to their favorite and specific menu, pathogens count on plant sucrose synthase (SuSy), a glycosyl-transferase enzyme (displaying invertase activity) that plays a key role in sugar metabolism pathways, catalyzing the reversible cleavage of sucrose into fructose and glucose, hence making it available in different host cellular compartments, including the intercellular apoplast space [27]. Therefore, representing a potential rice-blast susceptibility target, our shotgun proteomic analysis revealed *OsSUS4* (Q10LP3 - UniProt code), a sucrose synthase-like enzyme, 3.75 (log_2)-fold increased in the susceptible interaction at 12 hpi (comparison S_12 hpi/R_12 hpi). Aligned with our results, a notable enhanced expression and activity of plant invertase enzymes associated with fungus compatible interaction has been reported by several previous studies [28,29].

3.4. Keep calm, don't 'ROS-stress'

Plants have evolved intricate regulatory systems to control immune responses. Because activation of stress-induced response will likely cause deleterious effects on normal cell functioning, both positive and negative signaling pathways interplay to orchestrate and manage appropriate amplitude and duration of defense response [30]. A well-known characteristic observed in plant-pathogen resistant interaction outcome is the timely recognition of invading pathogen linked with a rapid mounting and effective activation of host defense mechanisms, such as the oxidative burst of reactive oxygen species – ROS [31]. In our study, the 'oxidoreductase activity' GO category showed to be a highly represented (21%) molecular function of the resistant interaction at the early stage of infection (12 hpi) compared to its mock-inoculated treatment (Supplementary Fig. S3 -B). Consistent with plenty of previous reports suggesting the key roles of peroxidase ROS-related proteins in rice early defense responses against *M. oryzae*, our study revealed that from all 20 differentially increased proteins identified in the comparison R_12 hpi/R_mock-inoculated, 7 were related to this protein family.

The enrichment analysis (Table 1) also supported our findings showing that "cellular response to oxidative stress" was the most significantly enriched GO term overrepresented among DAPs in the R_12 hpi/R_mock-inoculated comparison (p-value = 7.62^{-3}). Curiously we could not identify any DAP related to 'oxidoreductase activity' in the compatible interaction when compared to its mock-inoculated treatment (Supplementary Fig. S3-B). These facts led us to hypothesize about negative regulation of PTI/ETI host defense-related pathway upon *M. oryzae* infection by the modulation of a diverse protein set composed by four (4) promising susceptible proteins (DAPs identified in S_12 hpi/R_12 hpi comparison), showing a remarkable increase in the early stage of infection, and to be unveiled hereafter.

Despite representing the sole protein that was selected based on a single-peptide identification, Os*PIN1* (B9FCZ8 – UniProt code) had the fourth highest fold change increase (7.02 [log_2]-FC) on the proteome profiling analysis of susceptible/resistant comparison at 12 hpi. Moreover, a correlation between the protein and transcript levels was observed, both being upregulated (Fig. 4), which supports the reliable identification of this protein. It constitutes a peptidyl-prolyl cis-trans isomerase NIMA-interacting-1 protein, containing 235 amino acids with a molecular mass of 25 kDa. Peptidyl-prolyl cis-trans isomerases (PPIases - EC 5.2.1.8), also known as cyclophilins are enzymes that accelerate protein folding by catalyzing the cis-trans isomerization of proline amidic peptide bonds in oligopeptides. Despite its central roles in diverse biological processes, growing evidence has suggested PPIase activity to play, among others, two key roles in plant-pathogen susceptibility, either as an antioxidant, removing sources of ROS production in plant cells, or as a "molecular switch", activating effector proteins secreted by viruses, bacterial, and fungal phytopathogens during infection [32,33].

Another putative *S*-proteins identified was *OsSRP*-ZXA protein (Q75H81 – UniProt code), 4.07 (log_2)-FC increased in S_12 hpi/R_12 hpi comparison. Serine protease inhibitor (serpin) is a broadly distributed superfamily of protease inhibitors that are present across the kingdoms Eukarya, Bacteria, Archaea, and some viruses [34]. Serpins and their cognate proteases are responsible for the control of crucial physiological processes in all higher eukaryotes, including growth, development, and programmed cell death, the last, being imperative for survival during (a) biotic stress management [35]. In cereal crops, despite its well-established function in grain quality and disease resistance, serpin proteins seem to play also a relevant, yet under-investigated, role in host susceptibility upon attack by phytopathogens.

A well-known feature of disease resistance is the hypersensitive response (HR), a localized programmed cell death (PCD) phenomenon, prompted by the effector-triggered plant immune response (ETI) upon pathogen recognition, which detains the intruder at the invasion spot [36]. It is well described that not only HR/PCD mechanism is positively regulated by plant metacaspase proteins, but also that the last is specifically inhibited by serpin proteins, such as *AtSerpin1* [37]. Hence, these observations suggest a conserved function of serpins on HR/PCD regulation, ultimately protecting plant cells from excessive cell death induced by certain pathogens. An interesting study performed by Bhattacharjee and co-workers [38] demonstrated that rice serpin protein *OsSRP-LRS* (sharing the same UniProt code with our *OsSRP-ZXA*), is induced by inoculation of necrotrophic fungal pathogen *Rhizoctonia solani* and plays a crucial role in the negative regulation of rice plant cell death. It was also shown that *Arabidopsis thaliana* serpin protein *AtSRP4* (O48706 UniProt code - sharing a notable similarity with our serpin

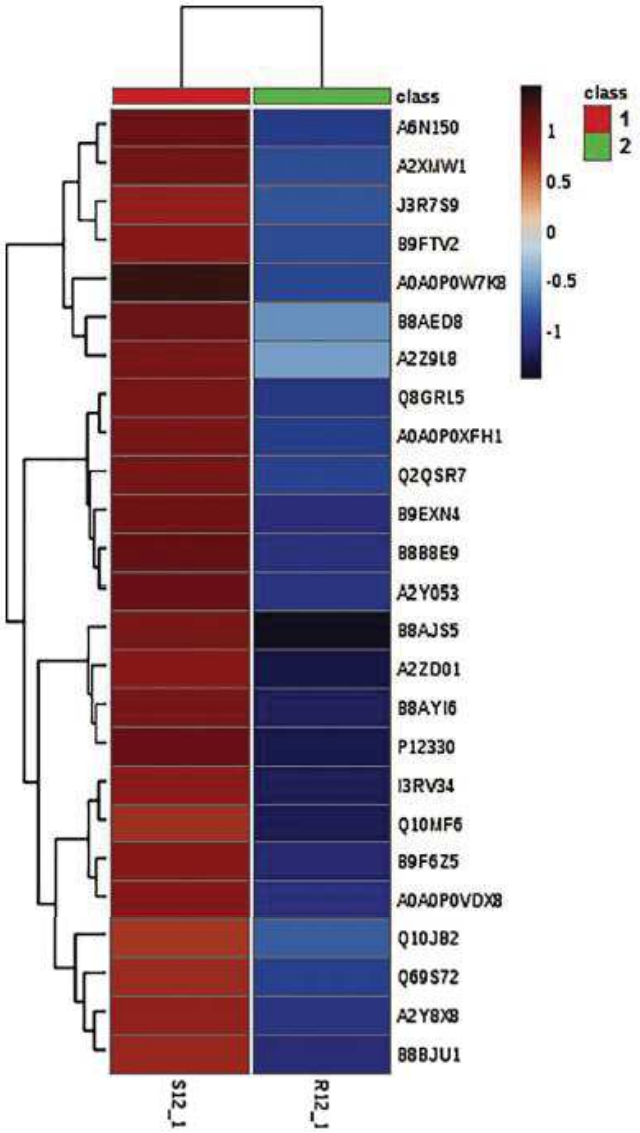

Fig. 3. Heatmap of 25 DAPs presenting the highest abundance contrast and lowest *p-values* among the 160 DAPs identified in the S12/R12 condition and grouped majorly in clusters # 4 and # 7. S12_1 and R12_1 represent biological replicates of each treatment; on the right, the ID of regulated proteins according to the UniProt database; class 1 and 2 represent the compatible and incompatible interactions, respectively. The analysis was performed using MetaboAnalist software.

Table 1
Summary of the GO enrichment analysis.

Funcional categories	Reference (*O. sativa* L.)	SS_12/S_mock			R_12/R_mock			S_12/R_12		
		#	p-value	+/-[a]	#	p-Value	+/-	#	p-Value	+/-
Total	43.569	1 out of 7			9 out of 20			42 out of 160		
Signal transduction	395	1	9.09E-03	+	0	1.00E-00	+	0	1.00E-00	+
Cellular response to oxidative stress	36	0	1.00E-00	+	1	7.62E-03	+	1	3.50E-02	+
Cellular metabolic process	1.702	0	1.00E-00	+	1	3.01E-01	+	9	2.98E-05	+
Stimuli cellular response	678	1	1.56E-02	+	0	1.00E-00	+	0	1.00E-00	+
Cellular homeostasis	33	0	1.00E-00	+	1	7.00E-03	+	2	5.28E-04	+
ROS metabolism	32	0	1.00E-00	+	1	6.80E-03	+	2	4.98E-04	+
Photosynthesis	40	0	1.00E-00	+	0	1.00E-00	+	2	7.61E-04	+
Sulphur-compound transport	9	0	1.00E-00	+	0	1.00E-00	+	1	9.59E-03	+
Hexose metabolism	56	0	1.00E-00	+	0	1.00E-00	+	2	1.45E-03	+
Gene expression	1.419	0	1.00E-00	+	1	2.58E-01	+	6	2.31E-03	+

[a] (+) over-represented and (−) under-represented GO terms.

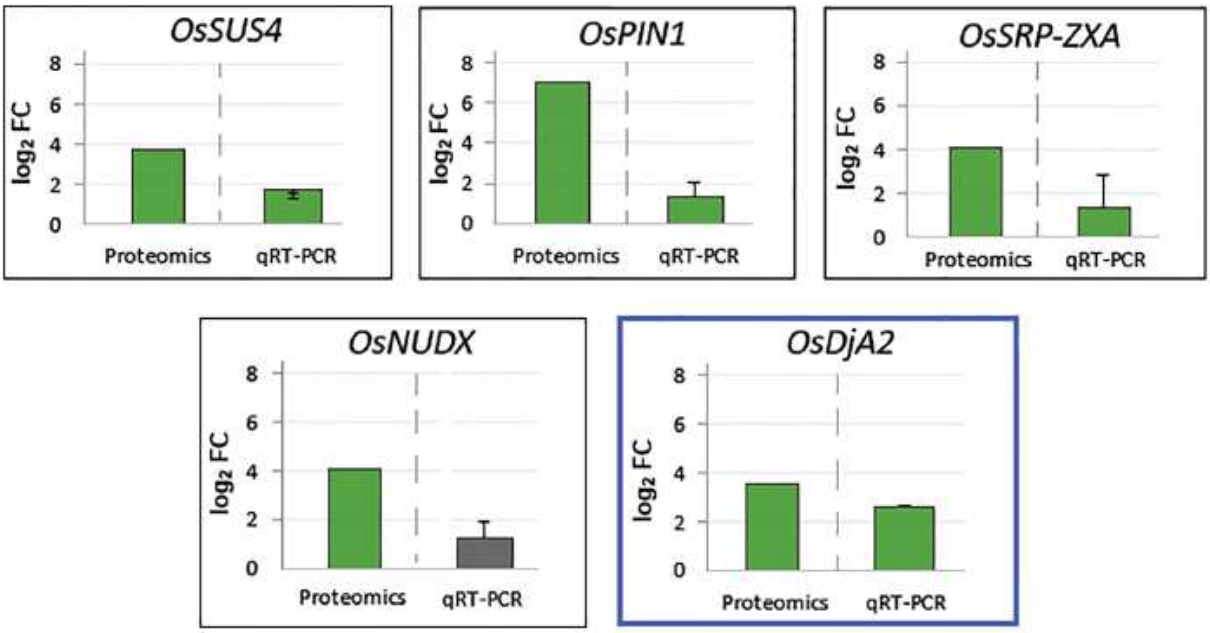

Fig. 4. Proteomics and RT-qPCR analysis of rice NILs – *M. oryzae* interactions. Y-axis represents the log_2-fold change of both differentially-increased candidate proteins identified by comparative shotgun proteomics between rice NILs IRBLi-F5 and IRBL5-M (susceptible and resistant to *M. oryzae* 9881 isolate, respectively) 12 hpi, and relative mRNA expression of cognate protein-encoded genes, determined by RT-qPCR in the same above-mentioned experimental conditions. The green bars represent both increased levels (up-regulated) of candidate proteins and relative mRNA expression. The gray bar represents the level of relative mRNA expression that did not show a statistically-significant regulation. The blue rectangle shows the greatest correlation between mRNA expression and protein increased levels. For qPCR experiments, the error bar represents the mean ± SE of three biological samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

OsSRP-ZXA protein) was induced by *Pseudomonas syringae* pv. *tomato* DC3000, reaching its highest peak of expression at 12 hpi [39]. Moreover, *AtSRP4* and *AtSRP5* (another serpin protein) knockout mutants exaggerated whereas overexpressing lines reduced plant HR-induced cell death. They also demonstrated that mutants of *AtSRP4* and *AtSRP5* suppressed the growth of bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 carrying the *AvrRpt2* effector. In this context, our hypothesis that rice plant (HR)-induced cell death responses are somehow manipulated in favor of *M. oryzae* installation/proliferation in its biotrophic phase, seems to be aligned with an increasing body of evidence that indicates the role of protease inhibitors as susceptibility factors in crop plant-pathogen interactions. However, the precise role of *OsSERPIN*-ZXA in rice-blast susceptibility remains to be characterized.

Another important revealed candidate to be addressed is *OsNUDX hydrolase domain-containing protein* (B8AZ64 – UniProt code). Showing the fourth-highest fold change among the DAPs identified in the comparison S_12 hpi/R_12 hpi, *OsNUDX* seems to be also strongly associated with rice-blast susceptibility. Nudix (nucleoside diphosphates linked to some moiety X) is a diverse superfamily of proteins, which are defined by the conserved Nudix motif amino acid sequence GX_5-EX_7-REVXEEXGU, widely distributed among all classes of organism ranging from viruses to humans [40]. Our search on RiceNetDB database (http://bis.zju.edu.cn/ricenetdb/) returned 29 genes encoding *OsNUDX* proteins in *Oryza sativa*. Despite a few studies addressing the effects of NUDX proteins in rice physiology, there are several relevant studies on *Arabidopsis thaliana* that suggests their role in susceptibility through the regulation of biotic stress plant responses. For example, not only the expression of *atNUDX7* protein in *A. thaliana* was shown to be induced upon attack of diverse phytopathogens, but also mutant plants (KO-nudx7) exhibited enhanced resistance to (a)virulent pathogenic strains [41,42]. It is well established that plant cells are equipped with a system of antioxidant enzymes involved in scavenging attacking radicals as well as in regenerating oxidized biomolecules, responsible for the sanitization of the cell plant environment. In this way, molecular and enzymatic studies have unambiguously reported that *AtNUDX7* protein plays a physiological role in maintaining cellular redox homeostasis during stress, by hydrolyzing excess NADH substrate (a potential source of superoxide) and preventing excessive stimulation of defense responses. *AtNUDT7* loss-of-function mutant plants displayed enhanced resistance to pathogens as well as increased levels of reactive oxygen species (ROS) and NAD(*P*)H on infected leaves [43,44]. Interestingly, some phytopathogens, such as the oomycete *Phytophthora sojae*, have developed a strategy to modulate plant defense response by secreting an effector that is in fact a NUDX enzyme [45]. Besides, this pathogen NUDX recruits a cyclophilin enzyme from its host plant to be activated and block plant ROS burst defense response.

To get a deeper insight into cellular redox states of infected rice NILs, we went through the proteomic profile comparison between compatible/incompatible interactions 12 hpi to check-out for DAPs ordinarily involved with cellular detoxification, such as peroxidase enzymes showing glutaredoxin/thioredoxin activities. As we expected, our results revealed a remarked differential increase of these antioxidant-related proteins, which ultimately abrogates a robust stimulation of host stress responses upon *M. oryzae* infection, reflecting the greater negative regulation of defense responses in the compatible interaction. In Table 2, we report a set composed of eighteen cell-protective antioxidant ROS-scavenger enzymes, identified at the early stage of infection, suggested here to be modulated by the fungus to 'keep a calm environment' for successful colonization.

A manual examination of differentially-increased proteins identified in the S_12 hpi/R_12 hpi comparison revealed an additional potential target protein involved in rice susceptibility to *M. oryzae*. Closing the specific set of highlighted susceptibility-related proteins revealed by our proteomics analysis is *OsDjA2* (Q6K850 - UniProt code), a molecular chaperone Hsp40/DnaJ family protein, which was 3.58 (log_2)-fold change increased in the susceptible interaction at 12 hpi. According to Sarkar and colleagues [46], the rice genome includes 104 DnaJ genes, categorized into four types based on the presence of specific conserved domains. Although only a few of these genes have been functionally characterized, the homologous protein-encoded gene OsDjA6 was

Table 2
Cellular homeostasis (ROS)-related DAPs.

Accession #*	Protein description	(log_2)_Fold change
		S_12 hpi/R_12 hpi
A0A0N7KJH5	Probable phospholipid hydroperoxide glutathione peroxidase [*Oryza sativa* Japonica Group]	6.383
Q6ES23	Probable lactoylglutathione lyase [*Oryza sativa* Japonica Group]	5.136
B7F6T2	Lactoylglutathione lyase-like [*Oryza sativa* Japonica Group]	4.574
B9EVP0	Peroxidase 2 - *OsPRX112* [*Oryza sativa* Japonica Group]	3.597
Q69TY4	Peroxiredoxin-2E-1 - *OsPRXIIe-1* [*Oryza sativa* Japonica Group]	3.529
O23877	Ferredoxin–NADP reductase, chloroplastic-like [*Oryza sativa* Japonica Group]	3.239
Q10GW1	*TAS* protein [*Oryza sativa* Japonica Group]	2.915
A2Z9L8	Probable glutathione S-transferase - *OsGSTu6* [*Oryza sativa* Japonica Group]	2.829
A2ZIK9	Monothiol glutaredoxin-S12 - *OsGRXs12* [*Oryza sativa* Japonica Group]	2.345
A2YL83	Thioredoxin-like protein - *OsCDSp32* [*Oryza sativa* Japonica Group]	2.240
A2XV46	2-alkenal reductase (NADP(+)-dependent) [*Oryza sativa* Japonica Group]	2.397
B8AJS5	Probable nucleoredoxin 1–2 - *OsNRX1–2* [*Oryza sativa* Japonica Group]	1.980
Q6ER94	2-Cys peroxiredoxin BAS1 - *OsBAS1* [*Oryza sativa* Japonica Group]	1.809
Q10AH9	Ankyrin repeat domain-containing protein 2A - *OsAKR2a* [*Oryza sativa* Japonica Group]	2.004
Q10SM7	Peroxisomal membrane protein 11–1-like - *OsPEX11–1* [*Oryza sativa* Japonica Group]	1.690
A2XU83	Glyceraldehyde-3-phosphate dehydrogenase A - *OsGPDH-A* [*Oryza sativa* Japonica Group]	2.593
Q2QMD4	Thiosulfate/3-mercaptopyruvate sulfurtransferase 2 - *OsSTR1* [*Oryza sativa* Japonica Group]	2.543
B0FFP0	Oxygen-evolving enhancer protein 2 - *OsOEE*, chloroplastic [*Oryza sativa* Japonica Group]	1.900

* UniProt accession code (available at uniprot.org/proteomes/).

recently proposed to act as a negative regulator in the rice PTI response through the inhibition not only of ROS accumulation, but also of the expression of defense-related genes in the SA-mediated pathway [47]. Interestingly, OsDjA6 (Q0JB88 - UniProt code) and OsDjA2 share a great similarity in terms of amino acid alignment and Hsp40 chaperone domain structures.

Altogether, our above-mentioned results reinforced the idea that both NILs underwent a substantial and distinct reprogramming of gene expression, clearly reflected in the resulting phenotypes of compatible/incompatible interaction (Fig. 1), notably during their initial responses to *M. oryzae* infection, a time point when the largest number of differentially increased proteins was identified. We also reasoned that rice NIL IRBLi-F5:*9881 M. oryzae* compatibility might be primarily prompted by the expression of those revealed rice putative susceptibility related proteins (*S*-proteins), strongly suggested to be modulated by the fungus to serve either as a nutrient source or as negative regulators of host PTI/ETI immune responses.

We also analyzed the expression levels of the five most promising *S*-candidates at 12 hpi in the compatible interaction and displayed the data together with their respective proteomics profile results (Fig. 4). The transcript levels of four *S*-candidate genes, namely *OsSUS4*, *OsPIN1*, *OsSRP-ZXA*, and *OsDjA2* were remarkably induced (upregulated) at this early beginning course of infection. Notably, *OsDjA2* showed the highest levels of either mRNA expression (2.55 log_2FC) and protein differential increase (3.58 log_2FC), thus representing the best target to our functional genomics assay. We further propose a model for the regulation of these candidate susceptibility proteins upon *M. oryzae* infection (Fig. 5).

3.5. Antisense technology for S-target validation

Transient-Induced Gene Silencing (TIGS) based on antisense oligonucleotide (ASO) consists of a robust and high-throughput system applied for gene function analyses [48]. Firstly demonstrated by Zamecnik & Stephenson [49], the concept of gene silencing by ASO is relatively straightforward: a user-tailored single-stranded synthetic DNA molecule (16–25 nucleotide long) designed to hybridize by virtue of Watson-Crick base-pairing with a complementary stretch of a target mRNA (sense strand) forming a heteroduplex that induces degradation of the transcript [50], usually by endonuclease RNase H-dependent cleavage mechanism, resulting in an effective gene silencing (knock-down). In the present study, we employed a phosphorothioate-modified oligo-deoxyribonucleotide (PTO)-based TIGS method (described in Lambertucci et al. [15], with modifications) for functional validation, *in planta*, of the engagement of our revealed targets in triggering rice-blast susceptibility. We selected *OsDjA2* as our most promising candidate for the control of blast disease by gene silencing.

To broaden our horizon of *S*-target prospection, we went through the results of the transcriptomic profiling of the same rice NILs-*M. oryzae* interactions [51]. Analyzing the differentially expressed genes (DEGs), we identified and selected two promising DEGs showing a remarkable increase in the compatible interaction. The first, *OsERF104* (LOC_Os08g36920), represents a member of the APETALA2/Ethylene Response Factor superfamily (AP2/ERF) well-known to regulate the expression of several rice genes related to stress response pathways [52]. AP2/ERF transcription factors have become the subject of intensive research activity in crops due to their involvement in a variety of biological processes and responsiveness to a wide (a)biotic stresses. For example, silencing of *OsERF922* rice gene by RNA interference (RNAi technology) enhanced rice resistance to *M. oryzae* [53]. Similarly, Wang et al. [54] reported a great enhancement of rice resistance to *M. oryzae* through CRISPR knock-out of the same *OsERF922* gene, asserting the role of this AP2/ERF domain-containing gene in rice-blast susceptibility.

The second transcriptome-prospected candidate, *OsPYL5* (LOC_Os05g39580), is a Pyrabactin Resistant/Pyrabactin Resistant-like (PYR/PYL) receptor or Regulatory Component of Abscisic acid (ABA) Receptor (RCAR). The binding of ABA phytohormone to PYL receptors triggers a phosphorylation cascade that activates downstream transcription factors promoting the expression of ABA-regulated genes and resulting in the acquisition of abiotic and biotic stress resistance in plants [55]. Aside from its well-documented roles in controlling plant physiological aspects and a wide variety of plant abiotic stresses, such as drought and salinity, ABA has also been implicated in modulating (positively or negatively) plant immune responses upon pathogen infection [56]. Furthermore, growing evidence is pointing toward ABA playing a pivotal role as a susceptibility factor not only in rice-*Magnaporthe* pathosystem but also in most plant-fungal interactions. Lastly, supporting the role of ABA-binding PYL receptors in crop plant-fungal susceptibility, Gordon et al. [57] showed that knockdown of wheat PYL4 receptor enhanced early resistance to *Fusarium graminearum* head blight (FHB) disease. Therefore, based on our *Omics* results, as well as in consonance with relevant literature findings we selected our three most prominent *S*-candidates: *OsDjA2*, *OsERF104* and *OsPYL5* to further validate their role in rice-blast susceptibility.

In the TIGS assay, at 16 h post-PTO infiltration, treated leaves of rice susceptible NIL IRBLi-F5 were atomized with *M. oryzae* spore suspension and foliar symptoms were visually examined and quantitatively assessed for healthy/lesioned ratio of foliar surface, 7 days post fungal inoculation (Fig. 6 I - II). Despite all treated plants showing typical lesion shapes of blast disease, rice leaves treated with PTO targeting *S*-candidates (Fig. 6 I A—C) clearly displayed slight lesions compared with control groups (Fig. 6 I D-E), which were entirely affected, presenting greater severity of blast disease. The quantification of symptoms (mean ± SE of

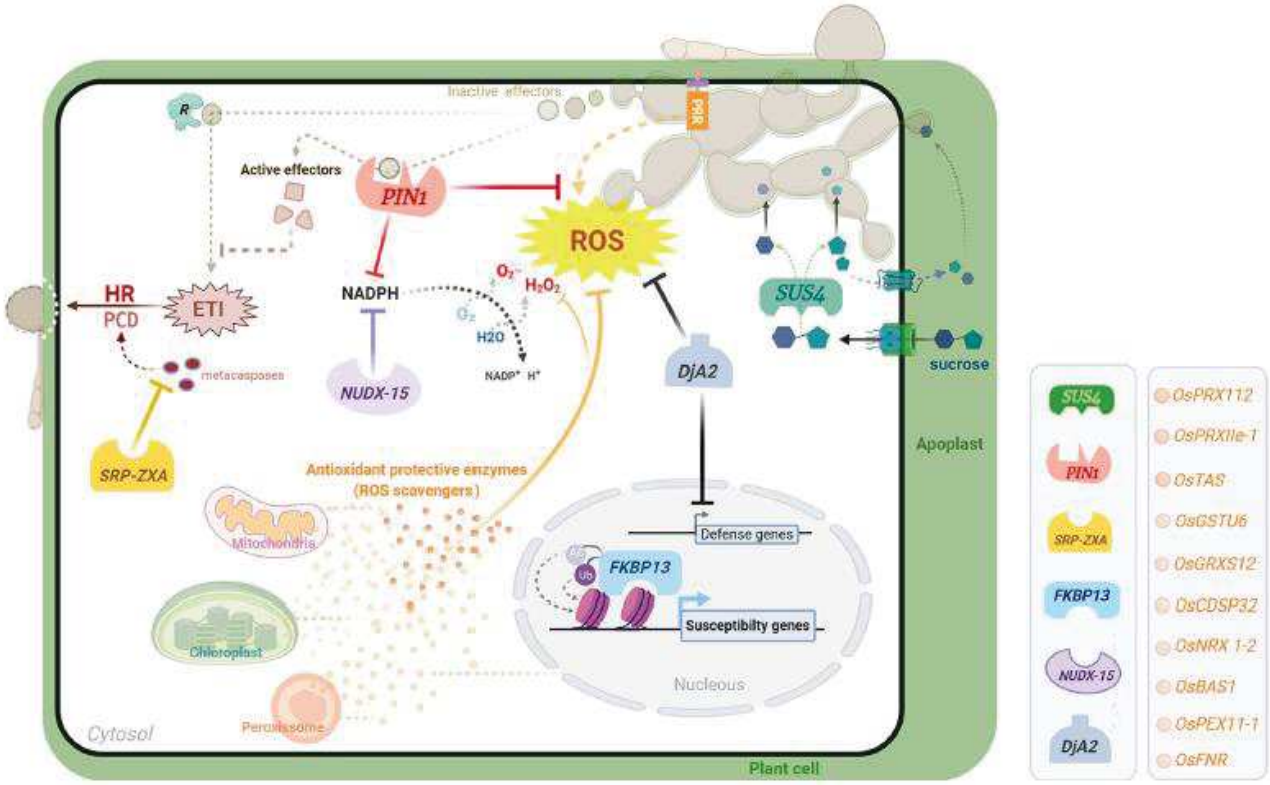

Fig. 5. A proposed model for the regulation of rice susceptibility responses upon *M. oryzae* infection. A set composed by six potential *S*-proteins (represented by colored enzyme-shapes and listed in a column at the right), conjoined with eighteen antioxidant ROS-protective enzymes (the ten most eminent are listed in a column at the right), presenting a remarkable differential-increase in the comparison S_12 hpi/R_12 hpi, suggested to be modulated by the fungus and thus associated to the compatibility state. Abbreviations: R: plant R-protein; PRR: pathogen-related receptor; ROS: reactive oxygen species; HR: hypersensitive reaction; PCD: programmed-cell death; ETI: effector-triggered immunity.

$N = 9$) in terms of the percentage of healthy foliar surface area, showed a notable increase of the healthy foliar tissue (up to 40%) on rice leaves treated with PTO targeting *S*-candidates in comparison with testimony group (treated only with *M. oryzae* fungus 9881 isolate) (Fig. 6 II). In addition, as expected, rice leaves treated with no-target negative control PTO (oligo designed to have no complementary sequence in rice transcriptome database), exhibited as much injury as the testimony group, assuring the reliability of the observed PTO-mediated phenotypes (Fig. 6 I A—C). Further analysis for significance of healthy foliar surface between PTO treatments and the testimony group (Fig. 6 II), pointed to statistical differences (post-hoc *t*-test; *p*-value <0.05) for all three PTO_*OsDjA2*, PTO_*OsERF104*, and PTO_*OsPYL5* treatments, ultimately suggesting an acquired resistance (partially, at least) to infection probably due to the knock-down of the corresponding rice susceptibility target transcripts. As we expected, the ANOVA between the no-target negative control PTO treatment and the testimony (only fungus 9881) showed a non-significant statistical difference. Although the reasons why plants have conserved and also promote the expression of such *S*-genes upon infection that would make them susceptible to pathogens remains a conundrum, it seems clear that modulation of cognate transcript levels is an important turning point for triggering rice-*M. oryzae* compatible state.

4. Conclusions

Taken together, our findings provided important molecular insights on rice-*M. oryzae* pathosystem at early stages of infection, disclosing potential rice susceptibility players with pivotal and specific roles in triggering a pathogen compatible state. Further, we propose different scenarios whereby the hemibiotrophic fungus *M. oryzae*, might succeed in causing disease by modulating host conserved *S*-targets involved, for example, with cell homeostasis (e.g.: antioxidant machinery), leading to a negative regulation of plant defense responses either by neutralizing ROS sources or hampering programmed-cell death triggering pathways. The pathogen can even hijack host *S*-targets responsible for making available favored fungus meals (fructose and glucose) in different host cellular compartments, hence, enhancing fungus survival, proliferation, and pathogenesis. Overall, we provide novel and alternative targets for innovative breeding strategies (e.g.: deletion of *S*-targets by CRISPR genome editing) toward the development of genetically improved crops with broader pathogen resistance.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2021.104223.

Data availability

All generated data comprising chromatography, mass spectrometry, protein quantification, and statistics, were deposited into the international repository *Center for Computational Mass Spectrometry* (University of California, San Diego) and into the *ProteomeXchange consortium*, under the IDs MSV000082657 and PXD010542 respectively.

Author contributions

FTPKT performed the experiments and wrote the manuscript. OBON performed plant growth and MMDFC the inoculations, WF, MC, and MVS performed the mass spectrometry analysis, RNM and RB performed the TIGS assay, OLF and AM designed and supervised the experiments.

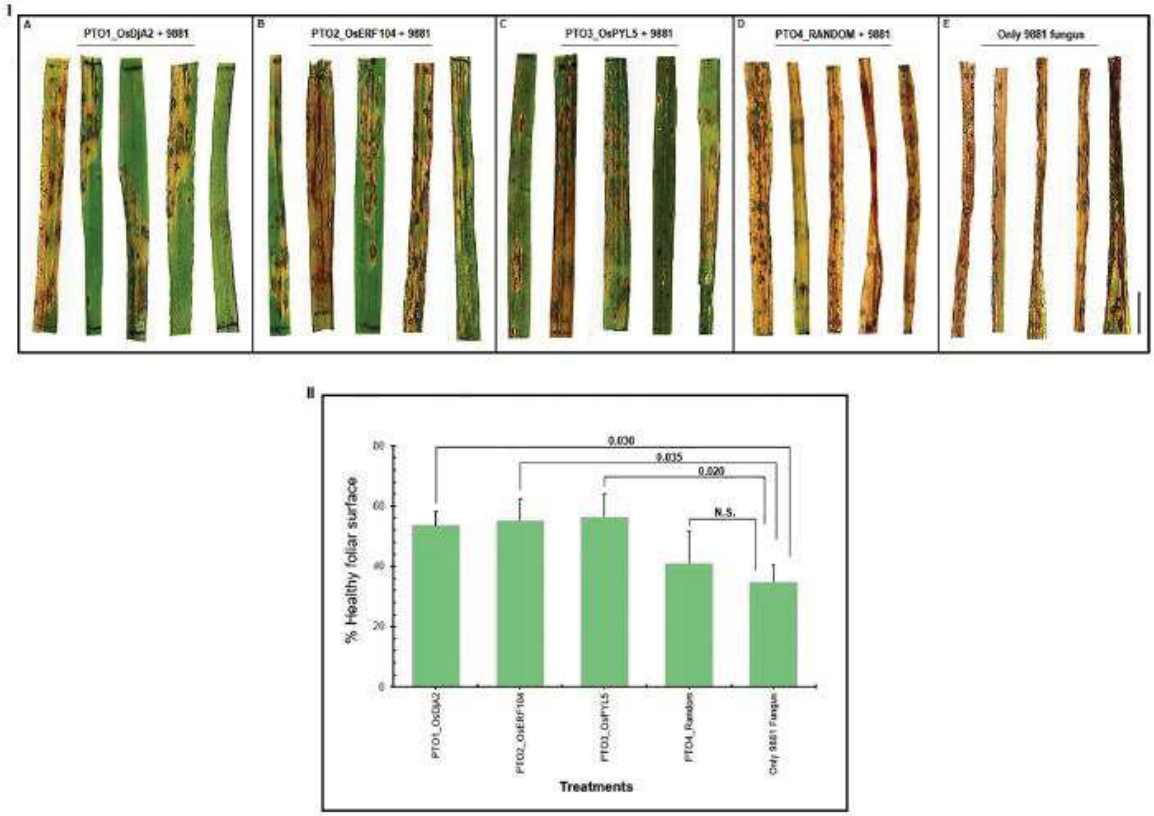

Fig. 6. Functional validation of potential *S*-target through PTO-based TIGS assay. Photographs were taken at 7dpi and severity of symptoms were analyzed using the software Quant®. I. (A-C) PTOs targeting rice-blast susceptibility candidate transcripts encoded by *OsDjA2*, *OsERF104*, *OsPYL4* rice genes, respectively; (D-E) random nonsense PTO as a no-target control treatment, and only fungus treatment, respectively. Scale bar = 1 cm. II. Significance for percentage of healthy foliar surface in relation to the control group (Only 9881 fungus) calculated with one-way ANOVA and post-hoc *t*-test ($P < 0.05$). Each bar represents the average of nine replicates (±SE). n.s., non-significative; PTO, phosphorothioate-modified oligodeoxynucleotides; NIL, near-isogenic line; dpi, days post-inoculation; ANOVA, analysis of variance.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial competing interest.

Data availability

Data will be made available on request.

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Supplementary figures

Figure S1 | Partial Least Squares Discriminant Analysis (PLS-DA) multivariate statistical analysis of the DAPs showing a clear grouping of replicates for each condition in a three-dimensional chart representing the three best discriminating components. Notice the dots representing S_12 hpi (green) and R_12 hpi (purple) among the most differentiated conditions.

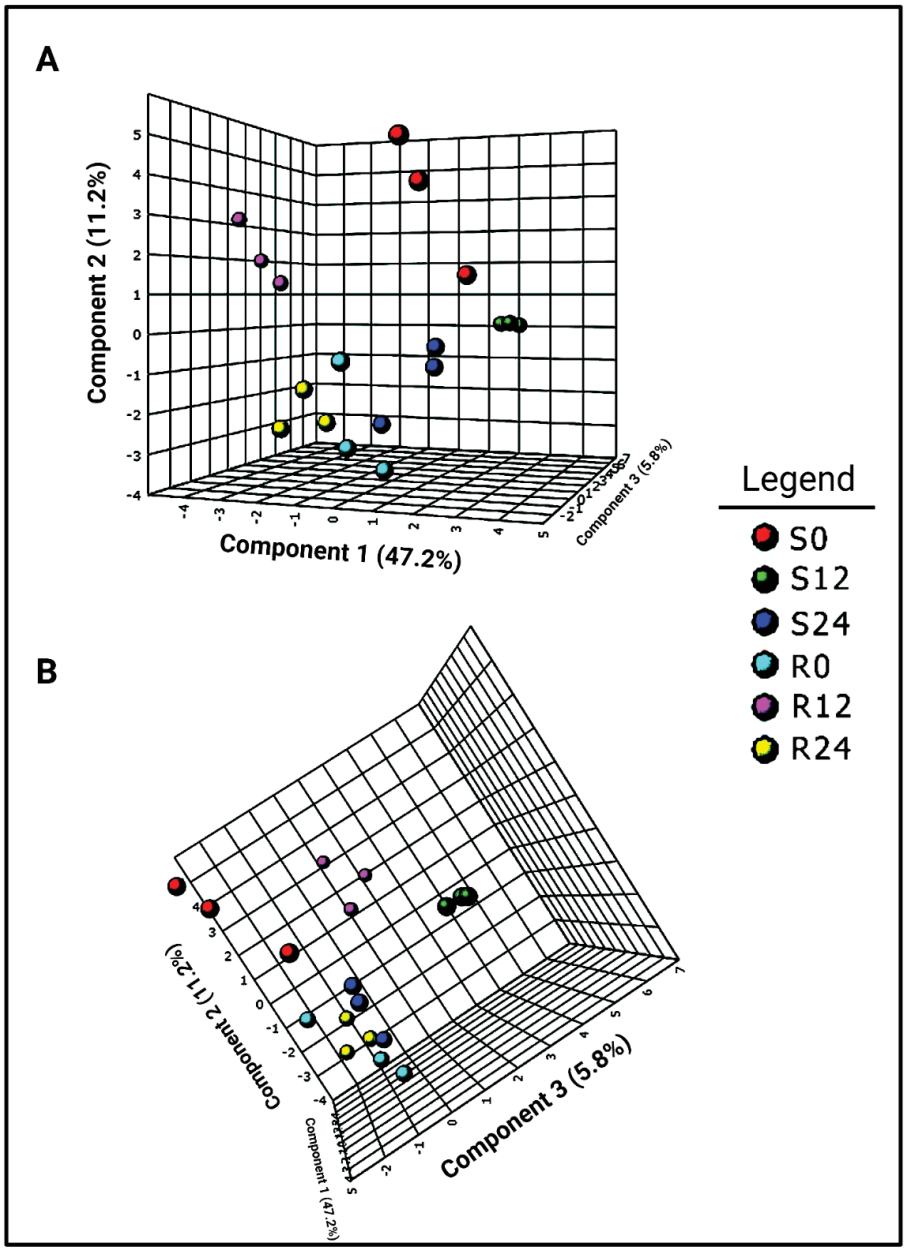


Figure S2 A-B | Clustering of total DAPs (n = 830) according to their relative abundance profiles between experimental conditions. (A) Dendrogram showing the grouping of regulated proteins according to their abundance profiles. (B) Total DAPs clustered from #1 to #7, according to the abundance profile of each protein regulated under conditions S0 (susceptible NIL mock-inoculated), S12 and S24 (compatible interaction 12, 24 hpi, respectively), R0 (mock-inoculated resistant sample), R12, and R24 (incompatible interaction 12, 24 hpi, respectively), represented by the 6 columns of the figure. The y-axis was transformed to show standard deviations (SD) from the mean. In other words, it shows how far the normalized abundance of a specific regulated protein is above or below the mean, after being divided by the SD of those data. Hence, a value of 0 would indicate that the run has exactly the mean value for that protein, and a value of +1 on the axis would represent a data point with a normalized abundance that is exactly one standard deviation above the mean of all runs for that protein. Analysis performed using Progenesis Qi software (.TIFF).

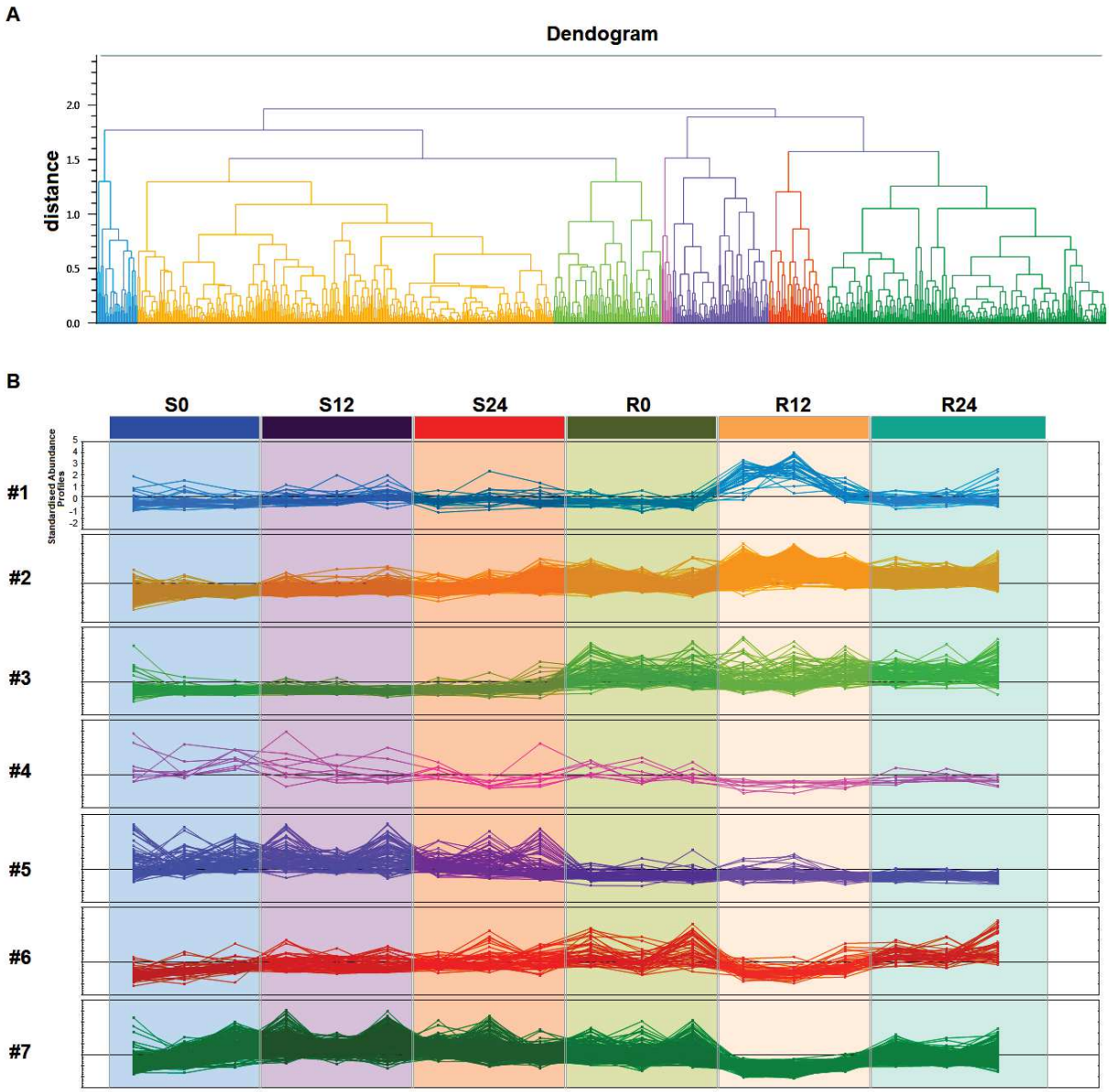
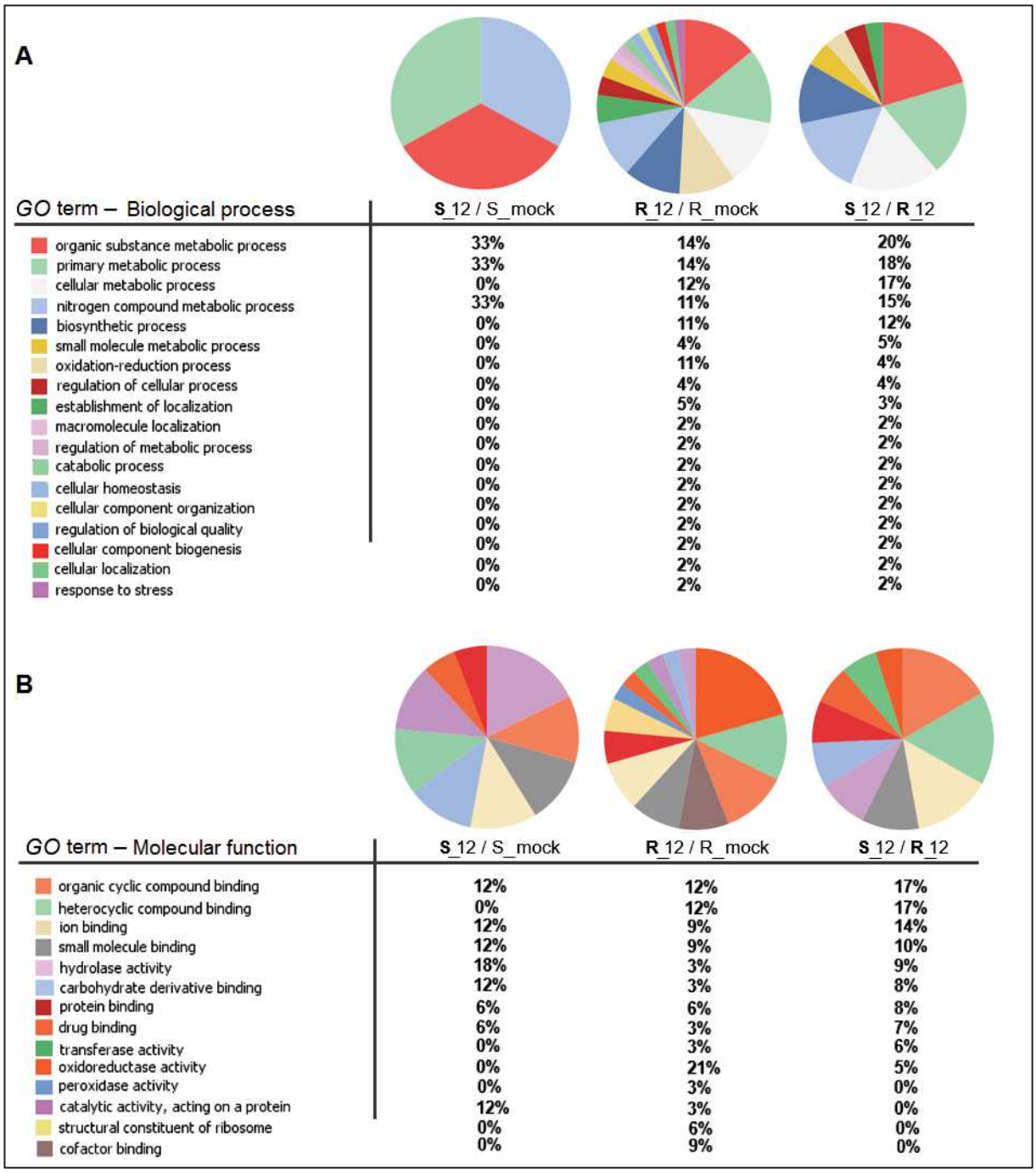


Figure S3 A-B | Functional categorization according to Gene Ontology (GO) of DAPs identified in compatible and incompatible interactions (comparisons S_12 hpi / S_ mock-inoculated; R_12 hpi / R_mock-inoculated; and S_12 hpi / R_12 hpi). GO terms are (A) biological process and (B) molecular function. Identified DAPs are represented here in percentage. The analysis was performed using Blast2GO software. (.TIFF).



GO term – Biological process	S_12 / S_mock	R_12 / R_mock	S_12 / R_12
organic substance metabolic process	33%	14%	20%
primary metabolic process	33%	14%	18%
cellular metabolic process	0%	12%	17%
nitrogen compound metabolic process	33%	11%	15%
biosynthetic process	0%	11%	12%
small molecule metabolic process	0%	4%	5%
oxidation-reduction process	0%	11%	4%
regulation of cellular process	0%	4%	4%
establishment of localization	0%	5%	3%
macromolecule localization	0%	2%	2%
regulation of metabolic process	0%	2%	2%
catabolic process	0%	2%	2%
cellular homeostasis	0%	2%	2%
cellular component organization	0%	2%	2%
regulation of biological quality	0%	2%	2%
cellular component biogenesis	0%	2%	2%
cellular localization	0%	2%	2%
response to stress	0%	2%	2%

GO term – Molecular function	S_12 / S_mock	R_12 / R_mock	S_12 / R_12
organic cyclic compound binding	12%	12%	17%
heterocyclic compound binding	0%	12%	17%
ion binding	12%	9%	14%
small molecule binding	12%	9%	10%
hydrolase activity	18%	3%	9%
carbohydrate derivative binding	12%	3%	8%
protein binding	6%	6%	8%
drug binding	6%	3%	7%
transferase activity	0%	3%	6%
oxidoreductase activity	0%	21%	5%
peroxidase activity	0%	3%	0%
catalytic activity, acting on a protein	12%	3%	0%
structural constituent of ribosome	0%	6%	0%
cofactor binding	0%	9%	0%

Chapter III:

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

CRISPR/Cas9-targeted Knockout of Rice Susceptibility Genes OsDjA2 and OsERF104 Reveals Alternative Sources of Resistance to Magnaporthe oryzae

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ABSTRACT

Rice blast, caused by *Magnaporthe oryzae*, is one of the most destructive diseases in agriculture leading to severe impacts on rice (*Oryza sativa* L.) harvests worldwide. CRISPR/Ca9 system has proven to be an effective tool for functional genomics revealing several host plant susceptibility genes as an attractive source for building plant resistance, thus contributing to rice improvement. 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 had also their function in plant susceptibility validated through gene silencing. Here, we report the CRISPR/Cas9 knockout of *OsDjA2* and *OsERF104* rice genes resulting in considerable improvement of blast resistance. Fifteen *OsDjA2* (62.5%) and seventeen *OsERF104* (70.83%) T0 transformed lines were identified from twenty-four regenerated plants for each target and were used in downstream experiments. Sanger sequencing revealed a reasonable percentage of homozygous mutation among *OsDjA2* (35%) and *OsERF104* (50%) CRISPR-edited lines. Phenotyping of homozygous T1 mutant lines revealed not only a significant decrease in the number of blast lesions, but also a reduction in the percentage of lesioned leaf area when compared with non-edited transformed control lines. Our results support CRISPR/Cas9-mediated target mutation in rice susceptibility genes as an effective and alternative breeding strategy for building resistance to blast disease.

Keywords: *Gene editing, Plant-pathogen interaction, Rice-blast resistance, S-genes.*

INTRODUCTION

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[1]. Unfortunately, rice plant has to deal with *Pyricularia oryzae* (teleomorph *Magnaporthe oryzae*), an hemibiotrophic fungus responsible for rice blast, one of the most ubiquitous and destructive diseases affecting rice production globally[2]. The cultivation of rice resistant varieties, harboring single or a couple of major *R* genes is the most used and environment-friendly approach to cope with *M. oryzae* infection[3]. Unfortunately, along with being a labor-intensive technique, conventional breeding aiming at *R* gene-mediated resistance is race specific and partially efficient. Moreover, resistance is broken down within few years after its commercial use[4].

However, alternatively to the resistance governed by *R* genes, the genetic manipulation of host susceptibility (-*S*) genes represents a powerful source towards a more resolute rice-blast resistance[5]. Although plant-pathogen arms race has forced pathogen to continuously evolve new strategies to evade or suppress plant immunity, most pathogens, especially hemibiotrophic fungus, such as *M. oryzae*, require host cooperation for the establishment of a compatible interaction, and typically exploit hosts' *S* genes to facilitate their proliferation[6]. Hence, all plant genes that somehow facilitate infection and/or support compatibility can be considered as a *S*-gene[7]. In the past, several case studies have already demonstrated the achievement of a more durable and broad-spectrum crop disease resistance by mutagenesis of *S*-genes. One of the best-known 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[8]. *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[9]. Further investigations of different pathosystems assisted by omics (e.g., proteomics and transcriptomics) together with gene silencing technologies (e.g., ASO,

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[10], 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[11].

In a previous shotgun proteomics study[12], 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 *M. oryzae*, and it had also its gene expression validated by qRT-PCR. Aiming to reinforce our set of candidate target-genes, as well as to broaden our frame of prospection, a second potential candidate was picked from a transcriptomics study performed by our group[13]. *OsERF104* (LOC_Os08g36920), was the most notable Differentially-Expressed Gene (DEG) (4.22 [$\log_2$]-fold change) identified 24 h post infection with *M. 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 notable decrease in foliar blast disease symptoms compared with control plants. Therefore, although the molecular mechanism of rice susceptibility to *M. 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.

Here, the CRISPR/Cas9-target KO of *OsDjA2* and *OsERF104* genes in the model japonica rice variety Nipponbare is reported. Homozygous mutant lines of T1 progeny carrying edited forms of each targeted gene displayed an enhanced resistance to blast disease.

MATERIAL AND METHODS

Plant materials and growth conditions

Rice cultivar Nipponbare (*Oryza sativa* L. spp. *japonica*) plants were grown in a containment greenhouse facility at Cirad, France, under the following conditions: temperature, 28°C day and 24°C night; 60% humidity. The natural light was complemented by artificial sodium light (700 µmol/m²/s). For blast inoculation, T1 progeny rice seeds from non-edited transformed plants recovered from the transformation experiment (hereafter named 'control lines') and homozygous mutant lines were sown in rows (20 seed per row) in 60 × 30 × 5 cm plastic seedling-nursing trays and maintained in a greenhouse optimal conditions at BGPI, France.

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

Gene-specific spacers (20nt long sgRNA templates) for each target *OsDjA2* and *OsERF104* were designed using CRISPOR-assisted website[14], available at http://crispor.tefor.net/. We then inserted the sgRNAs into an entry vector derived from Miao et al.[15]. Briefly, single-stranded gRNAs (20nt oligos) were synthesized as spacer-containing primers (Supplementary table S1), combined by annealing, and cloned into *BsaI*-digested pENTRY vectors for expression of each guide RNA. Subsequently, the resulting sgRNAs placed under the control of the Pol III U3 promoter, were cloned through LR reaction into the T-DNA region of a destination binary vector originally described by Miao et al.[15] and modified through replacement of the hpt gene by a castor bean catalase intron-containing version of the hpt gene. The T-DNA also carries a rice optimized Cas9 coding sequence controlled by the regulatory region of the maize ubiquitin 1 gene. The final CRISPR constructs for each target gene (*pCR_OsDjA2* and *pCR_OsERF104*) were confirmed by Sanger sequencing using specific primers (Supplementary Table S1). Potential off-target mutations caused by our designed gRNAs in CRISPR/Cas9-induced mutant plants were predicted by CRISPOR tool. For each of our target-genes, we designed specific primers (Supplementary Table S1) to amplify a genomic region (about 600 bp) flanking one

top-ranking off-target site showing 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 Zhang et al[16], with adaptations. Briefly, rice cv. 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 (4.5g/L phytagel) in rectangular (40cm x 30cm) Petri dishes. The seedlings were grown in the dark for 7 to 11 days at 26 °C in a growth chamber. Further protoplast isolation, purification, and transformation steps are detailed on the Supplementary Material.

Rice stable transformation

Rice transformations were carried out as previously described by Hiei et al.[17], with modifications. Briefly, *Agrobacterium tumefaciens* strain EHA105 were 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 WT 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 acclimatation 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 (T0) was extracted by MATAB (Mixed Alkyl Trimethyl Ammonium Bromide) method[18] and the presence of Cas9 in primary transformants and its segregation to the progeny was ascertained using the primers listed in Supplementary Table S1.

Transfer DNA (T-DNA) copy number was estimated by a DNA-based quantitative PCR (qPCR) optimized method using hpt-specific primers. Briefly, the isolated genomic DNAs were diluted to 0.5 and 5 ng / µL. A quadruplicate (4 reactions for 1 sample) qPCR was performed per CRISPR transformant line for the presence of hygromycin resistance gene and the reference gene (qPCR33, Actin-1, putative, expressed). A volume of 1 µL of gDNA was added to 0.3 µL of Forward primers (10 µM), 0.3 µL of Reverse primers (10 µM), 0.4 µL of H_2O, and 3 µL of SYBRgreen, q.s.p. 6 µL. The reaction and real-time fluorescence readings were carried out using a Light Cycler 480™ (Roche®) with the following PCR conditions: 5 min at 95 ° C; 45 cycles with (20 s 95 ° C, 15 s 60 ° C, 20 s 72 ° C); then 5 s at 95 ° C. A melting curve was performed with 1 min at 65 ° C and a continuous increase of 0.11 ° C / s up to 95 ° C to ensure the amplification of a single DNA. The copy number of transgene was estimated after normalizing the amount of DNA using the reference gene and the DNA from a T0 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 T0 plants harboring only one T-DNA copy were subjected to PCR using on-target specific primer pairs (Supplementary Table S1) 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[19] (available at http://crispid.gbiomed.kuleuven.be/) and CRISPR-mediated InDels on alleles of each mutant event were decoded. Lastly, we employed the ExPasy Translate tool[20] (available at https://web.expasy.org/translate/) 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[21] (Protein Variation Effect Analyzer) algorithm, developed by Institute Craig Venter (available online at 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 *M. oryzae*, the inoculation of rice blast fungus *M. oryzae* was performed as described by Xu et al.[22]. Briefly, *M. oryzae* isolate GY0011, virulent (compatible) to rice cv. 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 days in dark incubator at 25˚C, and then aerial mycelia were washed off by gentle rubbing with distilled water and paintbrush. The colonies were then successively exposed to fluorescent light for 3 days to induce sporulation at 25˚C. Conidia were harvested by softly scraping and flooding the medium surface with distilled water containing 0.01% Tween 20 detergent and the concentration of conidial suspension inoculum was adjusted to 5x 10^4 conidia.ml^{-1}. Rice seeds of one control line and three independent homozygous T1 mutant lines of each targeted-gene were sown in trays of 20×12×5 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 *M. 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˚C 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 6 plants of each mutant line, 6–7 days post-inoculation (dpi), using the software QUANT®, according to Vale et al.[23]. Statistical analysis was performed using one-way ANOVA (p-value <0.05) followed by post hoc two sample T-test (p-values <0.05, <0.01) for average comparison between mutants and control lines.

RESULTS

CRISPR/Cas9 design and ex-vivo assessment of sgRNAs gene-editing activity

For the generation of double-stranded breaks (DSB) at the target sites, one sgRNA targeting the first exon of each rice-blast susceptibility gene were designed (Fig. 1a). The sgRNAs were independently placed under the control of the rice Pol III U3 promoter in T-DNA vectors also containing a rice-optimized Cas9 sequence driven by the maize Ubiquitin 1 promoter (Fig. 1b). To evaluate the efficacy of our CRISPR vectors in generating DSB at target sites, rice protoplasts were independently transformed with both constructs (*pCR_OsDjA2* and *pCR_OsERF104*) and genomic DNA was extracted, purified and subjected to the T7EI enzymatic cleavage assay. The results showed that our expressing vectors exhibit suited gene-editing activity on the target sites of rice protoplasts DNA (Fig. 1c), hence, supporting their use for creating rice mutant plants.

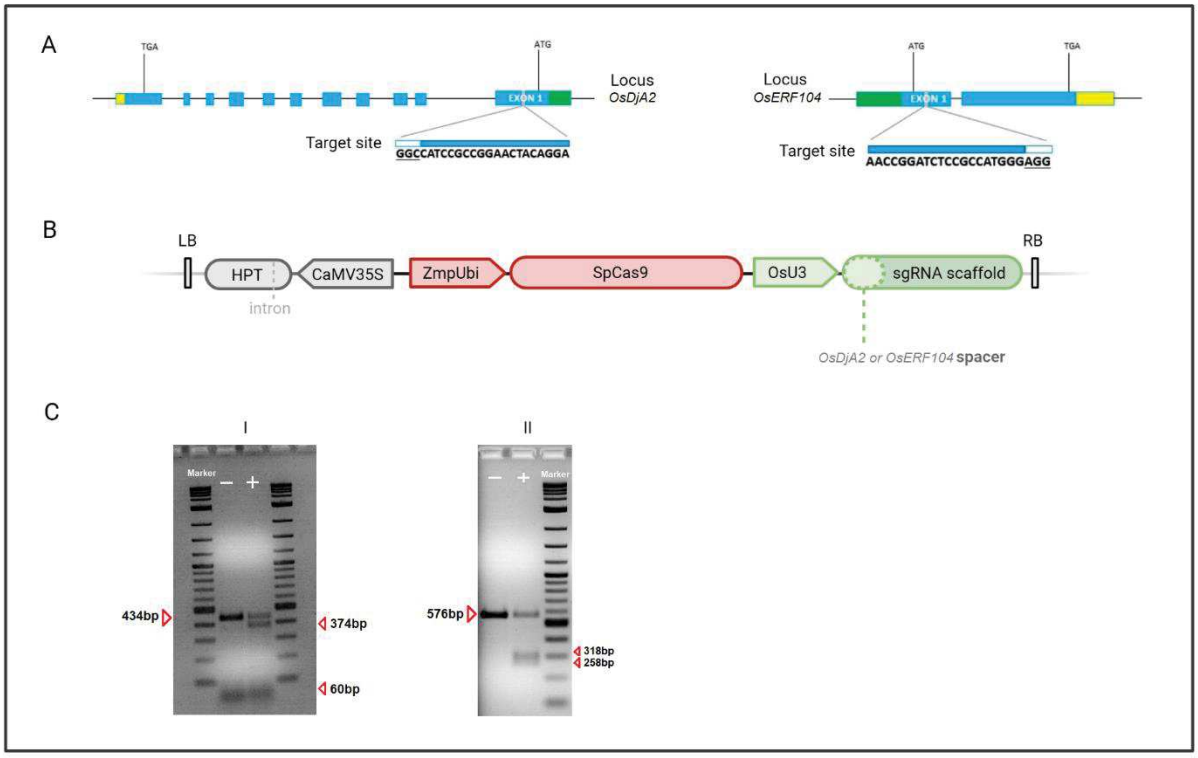


Fig.1 a-c: CRISPR/Cas9 design and T7EI assay for sgRNAs gene-editing activity. (A) A schematic map of gRNA target sites on genomic regions of OsDjA2 and OsERF104 loci. Exons are indicated as blue boxes, interspaced by introns shown as lines; PAM motif are underlined and represented as white boxes; ATG and TGA represents start and stop codons, respectively. (B) A simplified schematic representation of CRISPR/Cas9 T-DNA structure. (C) Assessment of gRNAs cleavage activity of rice protoplast

genomic DNA via T7EI assay. I – II represents 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.

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

Aiming to improve rice plant resistance to blast disease through CRISPR-KO of rice susceptibility genes, T-DNA binary constructs (*pCR_OsDjA2* and *pCR_OsERF104*) were used to transform mature seed embryo-derived calli of rice cv. Nipponbare by Agrobacterium-mediated genetic transformation. We obtained 24 primary transformant (T0) plants for each targeted-gene. A total of 23 (95.83%) T0 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 fifteen (62.5%) *OsDjA2* and seventeen (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: 93.33% and 70.59% of *OsDjA2* and *OsERF104* primary transformants, respectively, exhibited InDel mutations in the sgRNA target regions upon Sanger sequencing (Fig. 2a). Regarding the nature of CRISPR/Cas9-induced mutations, further examination of sequence chromatograms revealed that among *OsDjA2* targeted alleles, there were 8 (57%) harboring bi-allelic mutations, 5 (36%) homozygous, and 1 (7%) heterozygous. Likewise, among *OsERF104* mutant lines there were 5 (41%) harboring bi-allelic mutations, 6 (50%) homozygous, and 1 (8%) heterozygous (Fig. 2b). Aligned with literature reports on CRISPR/Cas9-mediated KO in rice[24,25], our results showed that the majority (62.5%, 20/32) of CRISPR/Cas9-induced InDels at both sgRNA cleavage sites were deletions of diverse lengths (-1 to -54), mostly observed in the bi-allelic mutant lines. As for the insertion mutations, single-nucleotide insertion (+ A), placed exactly three nucleotides upstream the PAM (i.e.: protospacer adjacent motif) sequence, prevailed (78.6%) on both *OsDjA2* and *OsERF104* homozygous mutant lines (Table 1).

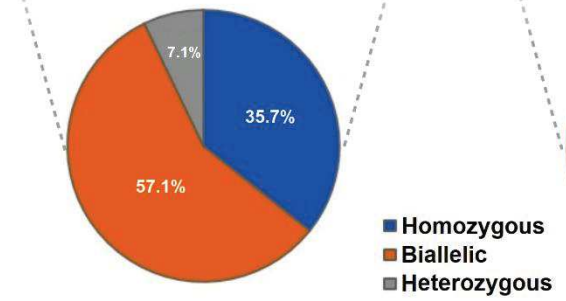
A

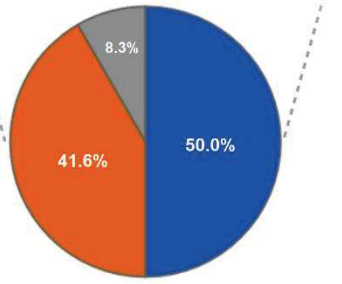
Targeted-gene	# of total T0 transgenic plants	# of total T0 transgenic plants harboring single (máx. two) copies of T-DNA	# of total T0 transgenic plants harboring site-mutations	Overall mutation efficiency (in percentage)
OsDjA2 (LOC_Os02g56040)	**24**	**15**	**14**	**93.33%**
OsERF104 (LOC_Os08g36920)	**24**	**17**	**12**	**70.59%**

B

Mutant genotype	# of mutated T0 plants (*OsDjA2*)
Homozygous	5
Biallelic	8
Heterozygous	1
Chimeric	0
Total	14

Mutant genotype	# of mutated T0 plants (*OsERF104*)
Homozygous	6
Biallelic	5
Heterozygous	1
Chimeric	0
Total	12





Homozygous
Biallelic
Heterozygous

Fig.2 a-b: The efficiency of CRISPR/Cas9-mediated genome editing of the target sites OsDjA2 and OsERF104, and the ratios of mutant genotypes in T0 mutant plants. Here, the overall mutation efficiency was calculated computing only the *OsDjA2* and *OsERF104* primary transformant plants containing only 1 (máx.2) transgene copies.

Assessment of InDel impacts on both ORFs and targeted-gene products

Functional validation of gene KOs was addressed by computational prediction of the induced-mutation impacts at the molecular level. *in-silico* outcomes of *ExPasy Translate tool* revealed that all *OsDjA2* and *OsERF104* homozygous T0 mutant lines exhibited a premature stop-codon very early on their ORFs (Supplementary Fig. S1), with primary impacts on the regular length of targeted gene-cognate expressed proteins, presumably caused by the frameshift mutations observed on both alleles in the sgRNA-target sites. The frameshift mutations observed on *OsERF104* T0 homozygous mutant lines (i.e.: +1[A]bp and +1[T]bp) resulted in an extensive

deletion of 185 amino acid (84% of the total protein content), vanishing with the whole transcriptional factor AP2/ERF domain, laying from 75 to 132 amino acid (Supplementary Fig. S2-A), and argued to be vital for protein function in (a)biotic stress regulatory networks[26]. Otherwise, the InDel mutation on *OsDjA2* T0 homozygous mutant lines (i.e.: -1[G]bp), despite generating great predicted deletion of amino acid content (77%), has conserved 90 amino acid 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') (Supplementary Fig. S2-B), both argued to be essential for dnaJ type protein function in the plant cell during stress conditions[28]. 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 T0 mutant lines, the large majority of observed amino acid deletions/substitutions were predicted as being deleterious (Supplementary Fig. S3), which means that despite *OsDjA2* remaining a truncated-protein, CRISPR/Cas9 mutagenesis probably led to the full knockout of the targeted-genes through loss-of-function (null) mutations.

Recovery of T1 progeny homozygous mutant lines

T0 mutant lines (*OsDjA2*_20.1 and _24.1; *OsERF104*_1.1, _5.1, and _6.1) harboring homozygous and predicted loss-of-function (null) mutations were selected, self-pollinated, and their progenies analyzed. We also generated T1 plants derived from T0 control lines (*OsDjA2*_27.1; *OsERF104*_14.1), obtained from the transformation process but with no mutations in the *OsDja2* and *OsERF104* genes. A total of 42 T1 plants (n=6 of each line) were firstly screened for the presence of T-DNA (Fig. 3). For all mutant lines, we observed that the identification and proportion (by around 1/3) of Cas9-free plants at T1 generation were in agreement with Mendelian segregation rate, since we have selected mutant parent lines harboring only one T-DNA insertion in the genome, previously estimated by qPCR.

Table 1: CRISPR/Cas9-induced InDel mutations at both OsDjA2 and OsERF104 sgRNA cleavage sites on primary T0 transformants.

Target gene: *OsDjA2* CCGGTAGGCGGCCTTGATGTCCT (WT sequence)			
Parent line (TO events)	**Estimated transgene copy number**	**Mutation description**	**Characterization of Indels on both alleles**
1.1	2	Heterozygous -19nt/WT	All1: cacctggcgc----------GCCTTGATGTCCTtgttgctcgcnn All2: gcgcaagcttCCGGTAGGCGGCCTTGATGTCCTtgttgctcgcnnn
9.1	2	Biallelic -2nt/-1nt	All1: gcgcaagcttCCGGTAGG--GCCTTGATGTCCTtgttgctcgcnnnnn All2: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnn
12.1	1	Biallelic -17/-1nt	All1: acctggcgcg----------GCCTTGATGTCCTtgttgctcgcnnnnn All2: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnn
13.1	2	Biallelic -1nt/-4nt	All1: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnn All2: gcgcaagcttCCGGTAG--------CCTTGATGTCCTtgttgctcgcnnnn
14.1	2	Biallelic +1nt/-1nt	All1: gcgcaagcttCCGGTA*T*GGCGGCCTTGATGTCCTtgttgctcgcnnn All2: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnn
15.1	2	Biallelic +1nt/-4nt	All1: gcgcaagcttCCGGTA*A*GGCGGCCTTGATGTCCTtgttgctcgcnnn All2: gcgcaagcttCCGGT----GGCCTTGATGTCCTtgttgctcgcnnnnnn
17.1	2	Biallelic -8nt/-4nt	All1: gcgcaagcttC----------GGCCTTGATGTCCTtgttgctcgcnnnnnn All2: gcgcaagcttCCGGTAG----CCTTGATGTCCTtgttgctcgcnnnnnn
18.1	1	Biallelic -1nt/+1nt	All1: gcgcaagcttCCGGTA-GCGGCCTTGATGTCCTtgttgctcgcnnnnn All2: gcgcaagcttCCGGTA*G*GGCGGCCTTGATGTCCTtgttgctcgcnnn
20.1	1	Homozygous -1nt	All1: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnnn All2: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnnn
21.1	2	Homozygous -2nt	All1: gcgcaagcttCCGGTAGG--GCCTTGATGTCCTtgttgctcgcnnnnn All2: gcgcaagcttCCGGTAGG--GCCTTGATGTCCTtgttgctcgcnnnnn
22.1	1	Biallelic -10nt/-1nt	All1: gcgcaagcttCCGGT----------GATGTCCTtgttgctcgcnnnn All2: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnnn
24.1	1	Homozygous -1nt	All1: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnnn All2: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnnn
26.1	1	Homozygote -15nt	All1: cctggcgcgc----------GGCCTTGATGTCCTtgttgctcgcnnnnn All2: cctggcgcgc----------GGCCTTGATGTCCTtgttgctcgcnnnnn
27.1	2	non- edited transformant	All1: gcgcaagcttCCGGTAGGCGGCCTTGATGTCCTtgttgctcgcnnnn All2: gcgcaagcttCCGGTAGGCGGCCTTGATGTCCTtgttgctcgcnnnn
27.2	1	Homozygous -3nt	All1: gcgcaagcttCCGGTAGGC---CTTGATGTCCTtgttgctcgcnnnnn All2: gcgcaagcttCCGGTAGGC---CTTGATGTCCTtgttgctcgcnnnnn

continued

Target gene: *OsERF104* CCTCCCATGGCGGAGATCCGGTT (WT sequence)			
Parent line (TO events)	**Estimated transgene copy number**	**Mutation description**	**Characterization of Indels on both alleles**
1.1	1	Homozygous +1nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn All2: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn
2.1	1	non- edited transformant	All1: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnn All2: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnnn
5.1	1	Homozygous +1nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn All2: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn
6.1	1	Homozygous +1nt	All1: ctcctggttgCCTCCCATTGGCGGAGATCCGGTTggtcatgtgannn All2: ctcctggttgCCTCCCATTGGCGGAGATCCGGTTggtcatgtgannn
8.1	2	Biallelic +1nt/-7nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn All2: ctcctggttgCCTCCCA----------GATCCGGTTggtcatgtgannn
9.1	1	Homozygous +1nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn All2: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn
12.1	1	Homozygous +1nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn All2: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn
14.1	1	non- edited transformant	All1: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnn All2: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnn
20.1	2	non- edited transformant	All1: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnnn All2: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnnn
22.1	1	Heterozygous -109 nt/WT	All1: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnnn All2: ctttcttctt----------tcgtcatcatnnn
25.1	1	non- edited transformant	All1: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnn All2: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnn
28.1	1	Homozygous +1nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn All2: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn
30.1	1	Biallelic +1nt/-20nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn All2: tactcctggt----------CCGGTTggtcatgtgan
31.1	1	non- edited transformant	All1: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnnn All2: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnnn
32.1	1	Biallelic +1nt/-1nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannn All2: ctcctggttgCCTCCC-TGGCGGAGATCCGGTTggtcatgtgannnnn
37.1	1	Biallelic +1nt/-23nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannnn All2: atgtactcct----------TTggtcatgtgan
38.1	1	Biallelic +1nt /-54nt	All1: ctcctggttgCCTCCCAATGGCGGAGATCCGGTTggtcatgtgannnn All2: ----------TTggtcatgtgan

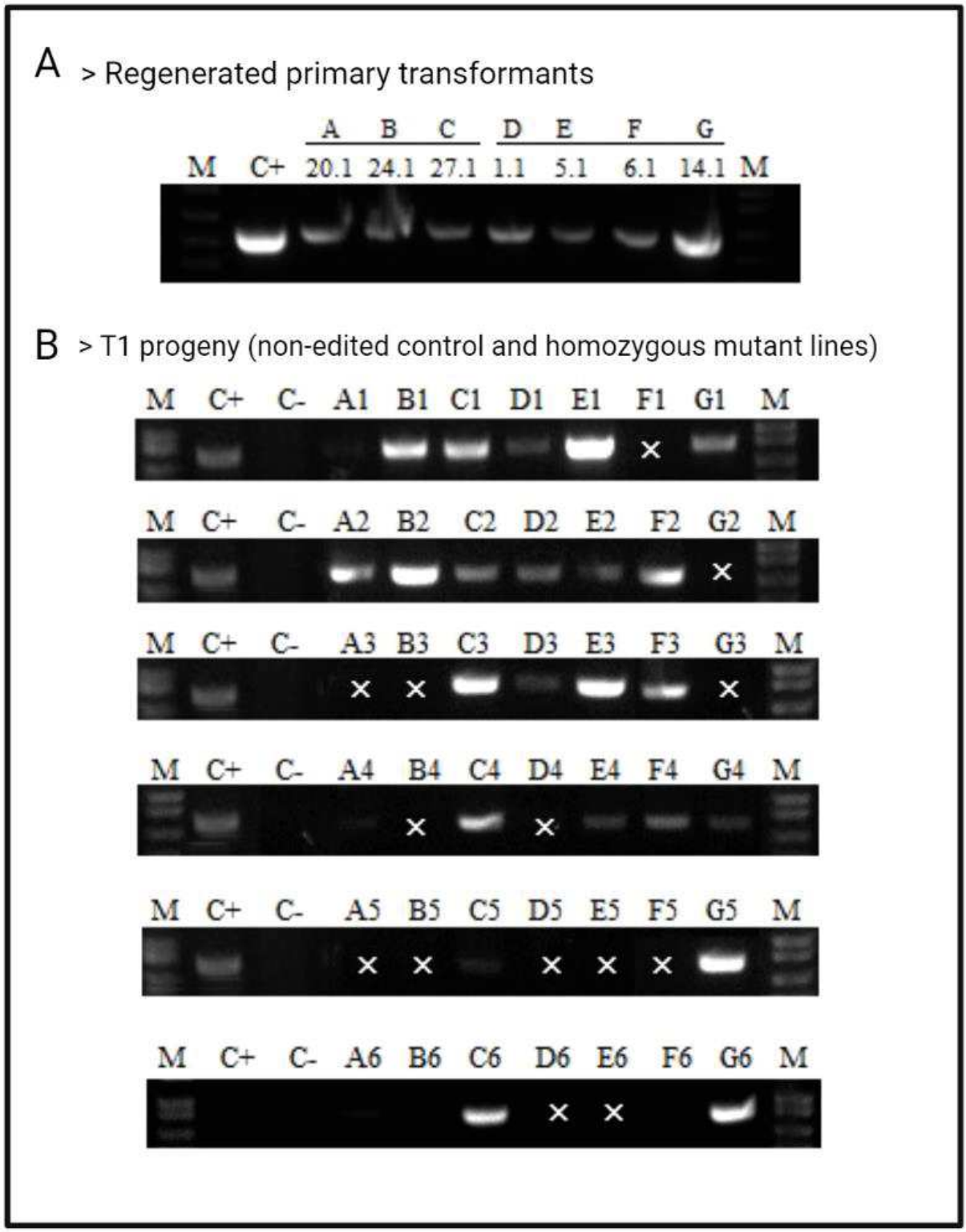


Fig.3 a-b: PCR-based screening for the presence of T-DNA in rice mutant plants. PCR products amplified from genomic DNA of (A) 7 independently T0 homozygous primary transformants OsDjA2_20.1, _24.1, _27.1, and OsERF104_1.1, _5.1, _6.1, _14.1 (A-C and D-G, respectively); (B) 42 T1 progeny plants (n=6 of each line), using specific Cas9 primer pair (see supplementary material). The letters (A-G) above the gel image refer to individual offspring (#1 to #6) of OsDjA2 and OsERF104 T1 lines. M: DNA molecular ladder; C+: CRISPR plasmid; C-: genomic DNA of WT rice cv. nipponbare; '×' indicates PCR negative for T-DNA.

In the case of the non-edited transformant control line OsDjA2_27.1, estimated to have two T-DNA insertion in the genome (see Table 1), all of its offspring (Fig. 3B: C1-C6) were PCR positive for T-DNA, without a clear segregation rate probably due to either multiple insertions or the small number of progeny screened. Cas9-positive and transgene-free T1 mutants were further genotyped by PCR and the on-target sites directly sequenced. As expected, all T1 mutant events were homozygous for the same mutations observed in both *OsDjA2* and *OsERF104* T0 parent lines (Table 2). Likewise, the non-edited transformed plants (control lines) are still 'wild-type', i.e.: without mutations in the sgRNA-target sites. It is worth noting that no unintended mutation was identified in the potential off-target loci of our homozygous T1 mutant lines (Supplementary Table S2). Therefore, we were able to recover a sufficient number of suitable control lines and homozygous mutant rice plants to subject to the blast resistance assay.

Table 2: Segregation of CRISPR/Cas9-induced InDel mutations in the sgRNA target regions of OsDjA2 and OsERF104 T1 progeny that were submitted to the phenotyping.

Target gene: ***OsDjA2*** CCGGTAGGCGGCCTTGATGTCCT (WT sequence)		
T1 Progeny (6 plants/line)	**Mutation description**	**Characterization of Indels on both alleles**
27.1_A1	non- edited transformant	All1: gcgcaagcttCCGGTAGGCGGCCTTGATGTCCTtgttgctcgcnnnnnnnnnnn All2: gcgcaagcttCCGGTAGGCGGCCTTGATGTCCTtgttgctcgcnnnnnnnnnnn
20.1_B1	Homozygous -1nt	All1: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnnnnnnnnn All2: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnnnnnnnnn
24.1_C1	Homozygous -1nt	All1: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnnnnnnnnn All2: gcgcaagcttCCGGTAG-CGGCCTTGATGTCCTtgttgctcgcnnnnnnnnnnn

Target gene: ***OsERF104*** CCTCCCATGGCGGAGATCCGGTT (WT sequence)		
T1 Progeny (6 plants/line)	**Mutation description**	**Characterization of Indels on both alleles**
14.1_D1	non- edited transformant	All1: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnnnnnnnnn All2: ctcctggttgCCTCCCATGGCGGAGATCCGGTTggtcatgtgannnnnnnnnnn
1.1_E1	Homozygous +1nt	All1: ctcctggttgCCTCCC*A*ATGGCGGAGATCCGGTTggtcatgtgannnnnnnnnr All2: ctcctggttgCCTCCC*A*ATGGCGGAGATCCGGTTggtcatgtgannnnnnnnnr
5.1_F1	Homozygous +1nt	All1: ctcctggttgCCTCCC*A*ATGGCGGAGATCCGGTTggtcatgtgannnnnnnnnr All2: ctcctggttgCCTCCC*A*ATGGCGGAGATCCGGTTggtcatgtgannnnnnnnnr
6.1_G1	Homozygous +1nt	All1: ctcctggttgCCTCCC*T*TGGCGGAGATCCGGTTggtcatgtgannnnnnnnnn All2: ctcctggttgCCTCCC*T*TGGCGGAGATCCGGTTggtcatgtgannnnnnnnnn

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

A total of 42 T1 control and homozygous mutant plants of each targeted-gene (i.e., n=6 per line: *OsDjA2*_27.1, _20.1, and _24.1; *OsERF104*_14.1, _1.1, _5.1, and _6.1), with no detectable vegetative development defects under normal growth condition, were tested for blast disease resistance. All plants at the fourth-leaf stage were inoculated with the fungal pathogen *M. oryzae* compatible isolate GY0011. At the 6–7 dpi, the number of blast lesions and the percentage of lesioned area on the fourth leaves of 6 plants of each mutant line were notably decreased in comparison with the control plants (Fig. 4 a-c). The quantification of both disease severity parameters was further evaluated using post-hoc Student's t-test which pointed to a statistical significance ($p < 0.05$*, < 0.01**) for the number of blast lesions on the leaves of OsDjA2_24.1, OsERF104_5.1 and _6.1 mutant lines (Fig. 4 d-e), and likewise for the percentage of foliar lesioned area on OsDjA2_20.1, OsERF104_1.1, _5.1, and _6.1 mutant lines (Fig. 4 f-g), in comparison with their respective control lines (OsDjA2_27.1, OsERF104_14.1). Although some events from the same mutant line (OsDjA2_20.1 and _24.1; OsERF104_1.1 and _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

Plant pathogen *M. 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[29]. To mitigate these negative impacts in agriculture, the usage of *R*-gene-containing cultivars have been for long time the most effective measure for rice crop protection against blast disease[30]. Nevertheless, dominant resistance governed by single *R* genes entangle several limitations[31]. 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.

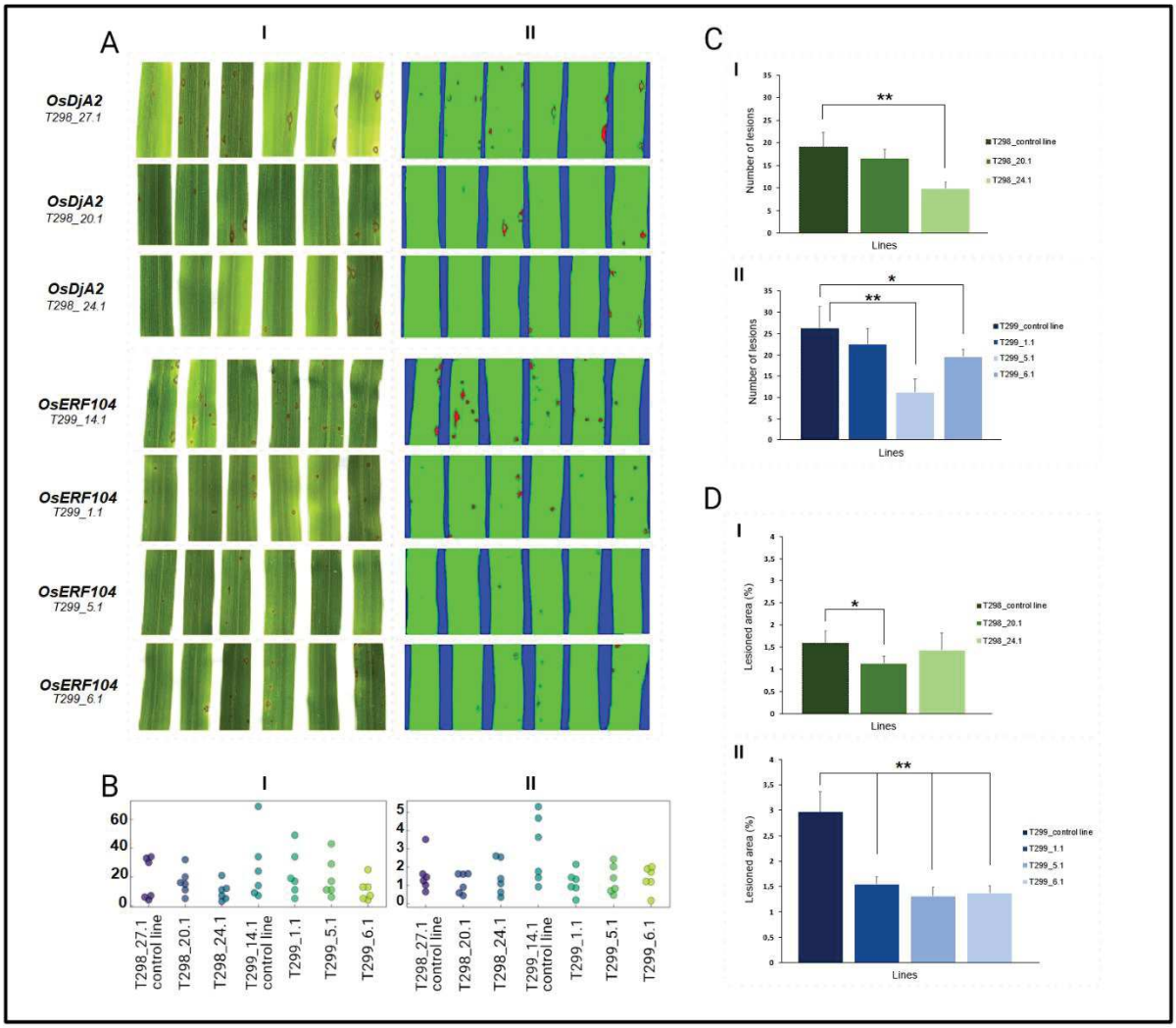


Fig.4 a-d: Identification of blast resistance in CRISPR/Cas9-edited rice mutant plants. (A) Blast resistant phenotypes of 42 T1 non-edited transformants (control lines) and homozygous mutant plants (i.e.: n=6 per line) of each targeted-gene (*OsDjA2*_27.1, _20.1, and _24.1; *OsERF104*_14.1, _1.1, _5.1, and _6.1). The whole fourth leaves of 6 plants (independently biological replicates) of each mutant line were detached from the inoculated plants at 6 – 7 dpi, scanned, and analyzed for the number of blast lesions (A-I) and the percentage of lesioned foliar area (A-II), using the software Quant®. The panels highlighted the blast symptoms at the central area of six independently rice leaves of each line. (B I-II) Swarmplot representation for the number of blast lesions and the percentage of lesioned foliar area, respectively, observed on each of the 6 leaves of all lines. (C I-II) Histograms showing the average of number lesions observed on the fourth leaves of 6 plants for each line of both OsDjA2 and OsERF104 targeted-gene. (D I-II) Histograms showing the average of percentage lesioned foliar area observed on the fourth leaves of 6 plants for each line of both OsDjA2 and OsERF104 targeted-gene. '*' indicates statistical significance (* $p < 0.05$; ** $p<0.01$, Two-sample T-test).

In the present study, we generated independent knockout mutant rice plants for *OsDjA2* and *OsERF104* putative rice-blast susceptibility genes by targeting its coding region using

CRISPR/Cas9 technology. 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, implying a prominent efficiency of CRISPR/Cas9-target mutagenesis. We obtained 5 OsDjA2 and 6 OsERF104 homozygous T0 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 and transgene-free T1 progeny. The presence of the same InDel mutations at the targeted-sites of T0-derived plants and also the expected Mendelian segregation ratios of 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 T1 mutant lines showing on-target frameshift mutations on both alleles, the most suitable mutant rice plants for the phenotyping. The inoculation with *M. oryzae* revealed an overall blast resistance for all tested mutant rice plants of both targeted-genes in comparison with the respective non-edited transformed control lines. In addition, the reduction of disease symptoms, especially in terms of the percentage of lesioned area, was significantly more pronounced (up to a half decrease) on the *OsERF104* gene KO plants, probably reflecting the ubiquity of AP2/ERF transcription factor in plant stress responses and its broader engagement with rice-triggered susceptibility. These results indicate that both *OsDjA2* and *ERF104* rice genes negatively regulates rice resistance to *M. oryzae*.

The present targeted genes *OsDjA2* and *OsERF104* were considered as a potential host *S*-genes in our previous comparative proteomics investigation of rice-*M. oryzae* interaction coupled to a transient-inducible gene silencing assay[12]. Their differential abundance (outstanding up to 4x [log]-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 to us their tight association with blast disease susceptibility. Accordingly, these observations find plenty support in literature.

Exposed to an ever-changing environment, deluged by biotic and abiotic stressors, plants must be able in maintaining cellular proteostasis for its proper growth, development, and survival[32]. This requires a fine-tune orchestration of a squad of molecular chaperones. Originally referred to as "Heat Shock Proteins" (Hsps)[33], 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 known as J-domain proteins (JDPs), are arguably the most ubiquitous components of the cellular chaperone network[34]. 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[35]. 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 J-domain, argued to be essential for the stimulation of the ATPase activity of Hsp70s[36]. 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 its conserved HPD motif, as well as 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 evidences that in viral pathogenesis, for example, the silencing of diverse J-domain-containing protein can lead to resistance or susceptible outcomes[37,38]. However, there are still large gaps on 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-*M. oryzae* pathosystem, consecutive studies have succeeded to functionally characterize the role of *DnaJ* homologues 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 and colleagues[39], where they reported the role of a chaperone DnaJ protein, *OsDjA6*, in the negative modulation of rice basal resistance upon *M. 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 *M. oryzae* in comparison with wild-type plants. More recently, Guojuan Xu *et al.*[40] reported a previously unidentified *M. oryzae*-infection tactics to trigger rice susceptibility. They found that MoCDIP4, an effector of *M. 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 *M. oryzae* infection starts to gather attention.

Another great player in the tangled modulation of plant immunity are 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 enemy's lifestyle and tactics of infection[41]. 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[42]. According with Muhammad Rashid *et al.*[43] exists 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[44]. Our CRISPR-edited rice gene *OsERF104* (generic name AP2/EREBP#152) is classified into the phylogenetic group IIIc of the rice ERF family[43,45], which is composed by 16 genes. The majority of its members has 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. It is noteworthy to emphasize that the present targeted gene *OsERF104* (LOC_Os08g36920), was selected as a potential *S*-gene candidate from a previous transcriptomics results, where it showed to be the most differentially-expressed gene (8.99 [log]-fold change; padj < 0.05) at 4 h post-infection (hpi) in the susceptible interaction (Bevitori *et al.*[13] – 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.*[13] – Table S3). Despite the fact that *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[46]. 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[47]. Further, increasing evidence indicating 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. Wenqin Lu et al.[48] 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[49]. Regarding rice-*M. oryzae* pathosystem, Dongfeng and co-workers[50] 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 promptly increase in the expression of pathogenesis-related (PR) gene products. Later, Wang F. *et al*.[51] reported a great enhancement of rice resistance to *M. oryzae* through CRISPR knockout of the same *OsERF922* gene, asserting the role of this AP2/ERF domain-containing gene in rice-blast susceptibility. Altogether, the appropriate manipulation of AP2/ERF TFs associated with negative regulation of plant immune responses has the potential to improve broad-spectrum disease resistance, thus representing high-value targets for genetic engineering and breeding of novel elite crops.

Our results revealed that CRISPR/Cas9-targeted KO of rice genes *OsDjA2* and *OsERF104* significantly enhanced resistance to *M. oryzae*, and also corroborates the findings of our previous work that suggested the ability of 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 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.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial competing interest.

Author contributions

FTPKT performed the majority of experiments and wrote the manuscript; AV and MP collaborated in the rice stable transformation; LH collaborated in the protoplast assay; DT performed the phenotyping assay; ACM, and CP collaborated with their rice genome editing expertise, kindly provided the CRISPR vectors, and conducted the experiments at the CIRAD facilities; OLF and AM designed and supervised the experiments.

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Appendix A. Supplementary data

The Supplementary data to this article can be found online at:

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Supplementary material

Fig. S1: *in-silico* outcomes of *ExPasy Translate tool*. The results indicate a premature stop-codon on the ORFs of homozygous T0 mutant lines a) *OsDjA2* (_20.1, _24.1); b) *OsERF104* (_1.1; _5.1), and c) *OsERF104* (_6.1), harboring the mutations -1[G]bp, +1[A], and +1[T], respectively. OsDjA2 protospacer (gRNA + PAM) is highlighted in orange; OsDjA2 protein J-domain and OsERF104 AP2/ERF protein domain are underlined in yellow; amino acid changes induced by frameshift mutations are indicated by gray boxes; stop-codons are indicated by red circles.

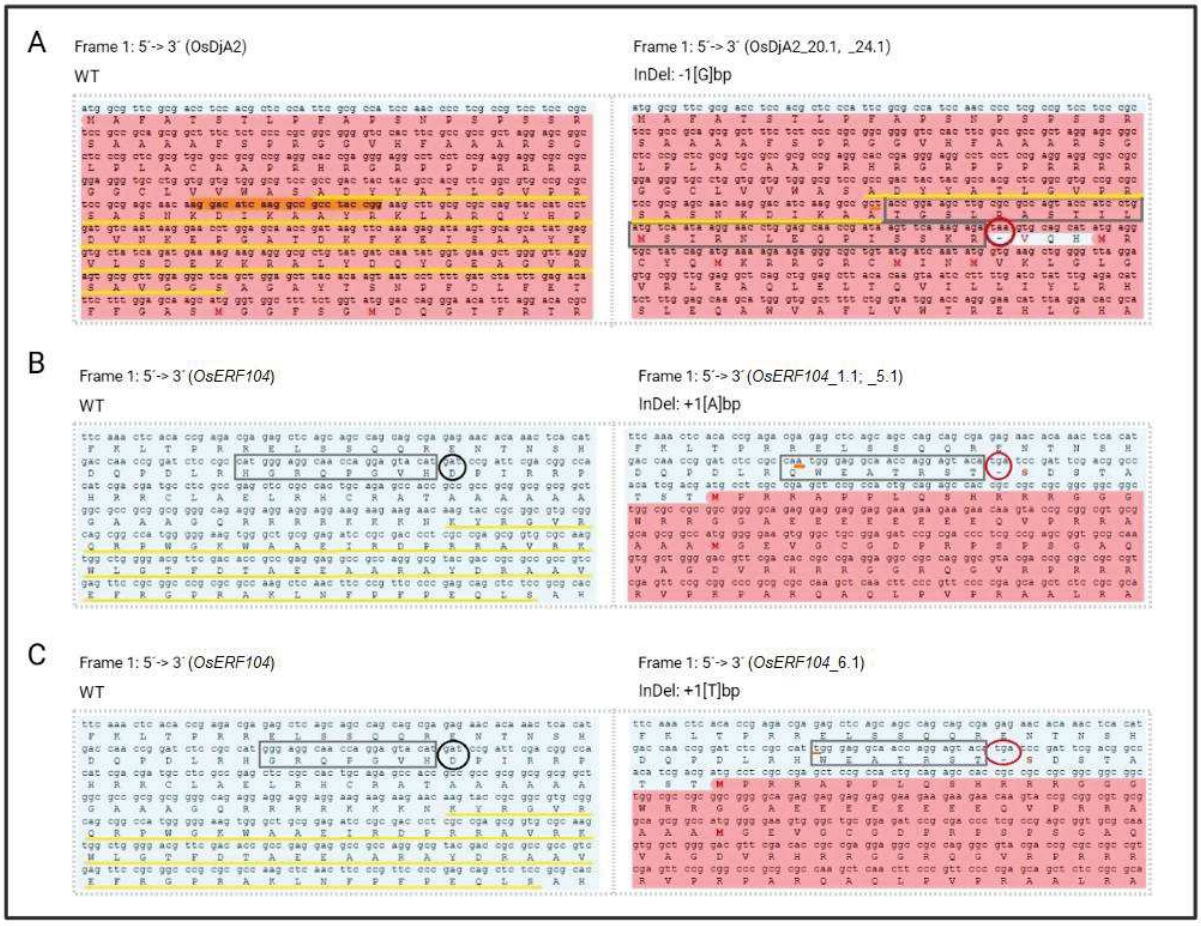
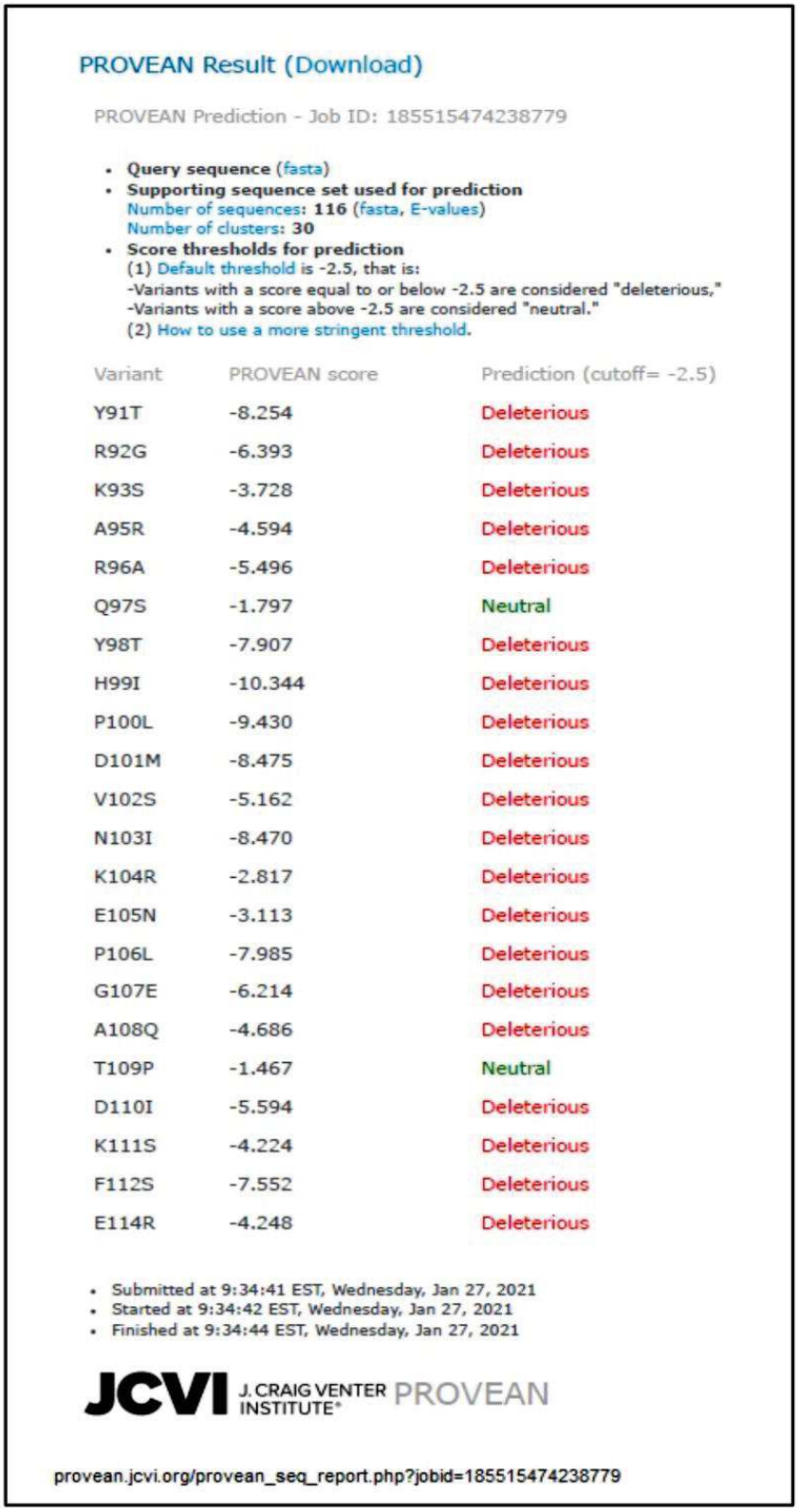


Fig. S2: InDel-induced frameshift mutations on the protein domains of *OsDjA2* and *OsERF104* T0 homozygous mutant lines. (A-B) UniProt access of OsDjA2 and OsERF104 wild-type (WT) protein sequences. (C-D) protein residues of homozygous mutant lines OsDjA2_20.1, _24.1, and OsERF104_1.1, _5.1, _6.1, derived from frameshift mutations caused by the InDels -1[G]bp, +1[A], and +1[T], respectively. DnaJ N-terminal conserved domain (called 'J' domain) of about 70 amino acids (70-146 aa), and AP2/ERF protein domain consisting in 60 amino acids (75-132 aa) are highlighted in red on both WT and remained protein sequences.

Fig. S3: *PROVEAN* (Protein Variation Effect Analyzer) scores for the query sequences of OsDjA2_20.1 and _24.1 homozygous T0 mutant lines, generated based on CRISPR-mediated InDel mutations. *Variant* means amino acid substitutions on the target protein sequence; *Prediction* means the impacts of InDel mutation (at the biological activity level) on the targeted-gene product.



PROVEAN Result (Download)

PROVEAN Prediction - Job ID: 185515474238779

- **Query sequence** (fasta)
- **Supporting sequence set used for prediction**
  Number of sequences: **116** (fasta, E-values)
  Number of clusters: **30**
- **Score thresholds for prediction**
  (1) Default threshold is -2.5, that is:
  -Variants with a score equal to or below -2.5 are considered "deleterious,"
  -Variants with a score above -2.5 are considered "neutral."
  (2) How to use a more stringent threshold.

Variant	PROVEAN score	Prediction (cutoff= -2.5)
Y91T	-8.254	Deleterious
R92G	-6.393	Deleterious
K93S	-3.728	Deleterious
A95R	-4.594	Deleterious
R96A	-5.496	Deleterious
Q97S	-1.797	Neutral
Y98T	-7.907	Deleterious
H99I	-10.344	Deleterious
P100L	-9.430	Deleterious
D101M	-8.475	Deleterious
V102S	-5.162	Deleterious
N103I	-8.470	Deleterious
K104R	-2.817	Deleterious
E105N	-3.113	Deleterious
P106L	-7.985	Deleterious
G107E	-6.214	Deleterious
A108Q	-4.686	Deleterious
T109P	-1.467	Neutral
D110I	-5.594	Deleterious
K111S	-4.224	Deleterious
F112S	-7.552	Deleterious
E114R	-4.248	Deleterious

- Submitted at 9:34:41 EST, Wednesday, Jan 27, 2021
- Started at 9:34:42 EST, Wednesday, Jan 27, 2021
- Finished at 9:34:44 EST, Wednesday, Jan 27, 2021

JCVI J. CRAIG VENTER INSTITUTE PROVEAN

provean.jcvi.org/provean_seq_report.php?jobid=185515474238779

Table S1: Primers used in this study

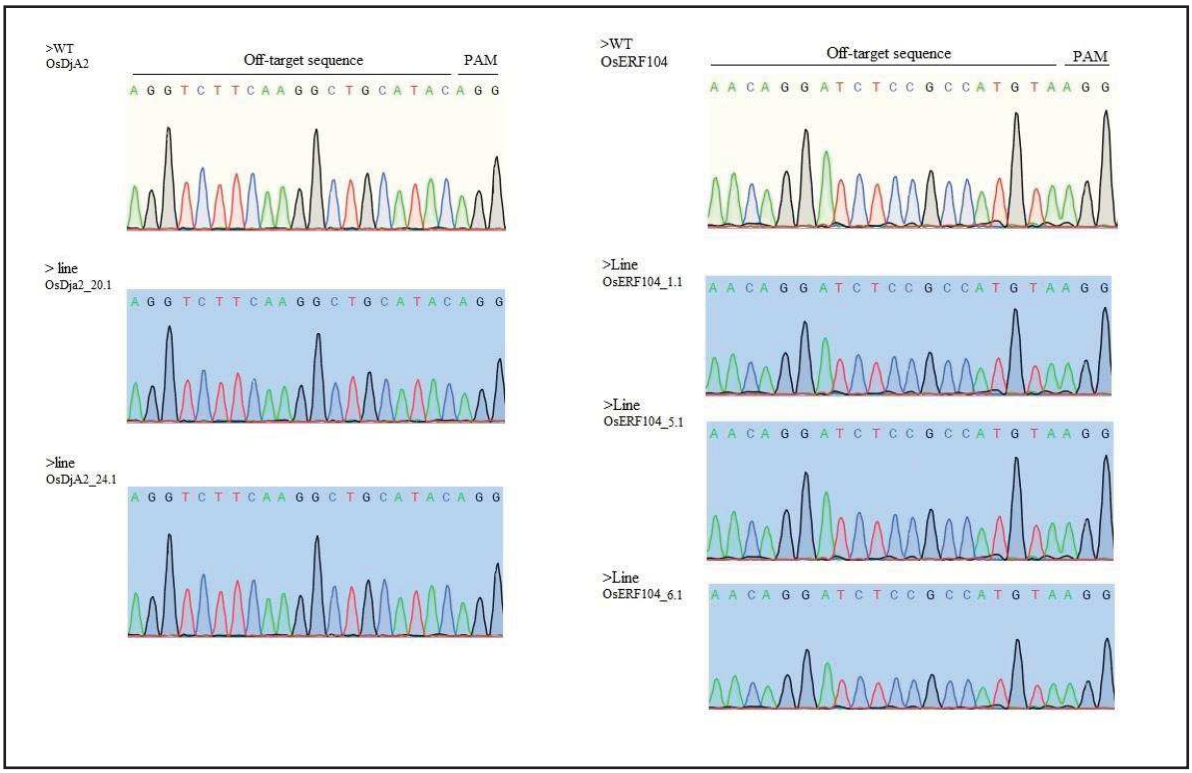
Primers	Primer sequence (5´→ 3´) *	Experiment
Dj_gRNA_F	**ggca**AGGACATCAAGGCCGCCTAC	Single-stranded oligos for OsDjA2 gRNA assembling and vector construction
Dj_gRNA_R	**aaac**GTAGGCGGCCTTGATGTCCT	
ERF_gRNA_F	**ggca**AACCGGATCTCCGCCATGGG	Single-stranded oligos for OsERF104 gRNA assembling and vector construction
ERF_gRNA_R	**aaac**CCCATGGCGGAGATCCGGTT	
M13_R	CAGGAAACAGCTATGAC	pENTRY vector sequencing
pDE-verif-LR-F	TGCAATGCTCATTATCTCTAGAG	pDESTINATION vector sequencing
on_Dj_F	AGGCCTTCTCAGATGTGCAC	Amplification of fragment across OsDjA2 on-target site
on_Dj_R	AGAAGCTTCTAGAAGGCGCA	
on_ERF_F	AATCCCATTATTCCGCCGCT	Amplification of fragment across OsERF104 on-target site
on_ERF_R	TCCTGTAGCCGGAGATGACA	
off_Dj_F	AGCATTTGCAGGCAAGTTGT	Amplification of fragment across OsDjA2 off-target site
off_Dj_R	TTAGCACCCTGTCGAACAACC	
off_ERF_F	GTACCCAACCTGCCACTCTC	Amplification of fragment across OsERF104 off-target site
off_ERF_R	TGAGGCGGTGACAACAACAAC	
Cas9_F	GAAGTACTCCATCGGCCTCG	*Cas9* (T-DNA) detection
Cas9_R	CGAGGAGATTGTCGAGGTCG	

***** Spacer-containing primers with overhangs (highlighted in bold - **ggca**/**aaac**) compatible with the pENTRY vector used in the study.

Table S2. Analysis of unintended-induced mutations on putative off-target sites of homozygous T1 mutant lines *OsDjA2*_20.1, 24.1; *OsERF104*_1.1, 5.1, and 6.1. For each of our targeted-genes one top-ranking off-target site predicted by CRISPOR tool was PCR amplified and analyzed by sequencing. PAM sequence is indicated in bold. Base mismatches in relation to the wild-type target sequences are marked in lower case. Chromatograms revealed no mutations in the off-target sites.

Target gene	Putative off-target site		No. of mismatches*	State
	Locus	Sequence		
OsDjA2 (LOC_Os02g56040)	LOC_Os02g54000	AGGtCtTCAAGGCtGCaTAC **aGG**	4	Non-mutated
OsERF104 (LOC_Os08g36470	LOC_Os08g36470	AACaGGATCTCCGCCATGta **AGG**	3	Non-mutated

*without taking into account those located in the first position of the PAM sequence (NGG motif)



Supplemental Material and Methods

Rice protoplast for ex-vivo editing assay

For protoplast isolation, healthy 9 days rice seedlings were used. A bundle of 100 etiolated stems were softly chopped into fine strips approximately 1.0-2.0 mm in length using sharp razors. The strips were immediately placed in 20ml of 600mM mannitol solution for 10min in the dark and then transferred into an enzyme solution (1.5% Cellulase RS, 0.75% Macerozyme R-10, 0.6 M mannitol, 10 mM MES buffer [pH 5.7], 7.5 mM $CaCl_2$, and 0.1% BSA). After 4 h digestion with gentle shaking (40 rpm) in the dark, an equal volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2mM MES buffer) was added, followed by shaking for 20 sec. The protoplasts were released by filtering through 30 μm nylon meshes into round bottom tubes and were washed twice using 15ml of W5 solution. The pellets were collected by centrifugation in swinging buckets at 250 g for 10 min (acceleration and deceleration = 2), re-suspended in 2ml of W5 solution, and followed a purification step.

Isolated protoplasts were purified passing the suspension through an increasing gradient of Percoll solutions (Percoll + W5 solution in four different concentrations: 10%, 20%, 40%, 60%). After centrifugation at 250 g for 10 min, the waste deposition phase (between Percoll gradient layers 2 and 3) was eliminated by pipetting and the tube softly inverted to mix the sample. The purified protoplasts were washed with W5 solution and pelleted by centrifugation. Finally, protoplasts were re-suspended in 4 ml of MMg solution (0.4 M mannitol, 15 mM $MgCl_2$, 5 mM $CaCl_2$, and 4 mM MES buffer) and counted under the microscope using a hemocytometer. After, protoplasts were diluted to a density of 2×10^5 protoplasts/ml and stabilized at least for 1 h on ice and in the dark before PEG-mediated transfection.

Protoplast transformation was carried out in a poly-ethylene glycol (PEG) solution [40% (W/V) PEG 4000, 0.2M mannitol, and 0.1M $CaCl_2$]. CRISPR expression vector (10 μg) was mixed with 200 μl protoplasts and 200 μl freshly prepared PEG solution, and the mixture was incubated at room temperature for 15 min in the dark. In parallel, we transformed protoplast

cells with a plasmid DNA fused to GFP that served to address the transformation efficiency and also as a 'wild-type' control in further T7EI enzymatic target cleavage assay. After incubation, 950 µL of W5 solution was added slowly, and the protoplast cells were harvested by centrifugation at 250 g for 10 min. The protoplast cells were resuspended gently in 1 mL WI solution (0.5 M mannitol, 20 mM KCl, and 4 mM MES at pH 5.7) and cultured in darkness at 27°C for 24 h.

The genomic DNA of transformed protoplast cells was extracted by MATAB (Mixed Alkyl Trimethyl Ammonium Bromide) method[17] and followed a purification step using QIAGEN™ *QIAamp DNA Micro*® Kit. The genomic DNA from protoplasts transformed with CRISPR vectors (and also with the 'control' GFP-plasmid) were subjected to the T7EI enzymatic assay, using NEB™ EnGen Mutation Detection® Kit, to access the ability of CRISPR constructs (Cas9-sgRNA ribonucleoprotein complex) in cleaving the target-genes *OsDjA2* and *OsERF104*.

Supplemental References

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General discussion

One of first intentions of the thesis was to gather elemental and *up-to-date* information about this breakthrough genome editing technology, CRISPR/Cas system. In the presented review manuscript, we focused among others, in recent findings and potential applications of CRISPR/Cas in improving major traits in agricultural plants, addressing both basic and applied researches. We found that for our target crop plant, although we do have several valuable studies focusing mostly on rice yield [Chapter I, pages 4-5], we are still lacking studies aiming to build a more resolute rice resistance against phytopathogens such as *M. oryzae* fungus, one of the main biotic constraints for rice paddy expansion worldwide, jeopardizing global food security. Nevertheless, CRISPR/cas toolbox seems to holds a precise tool to handle such serious problem, by the usage of Cas9-type enzyme (one of the most ordinary and spread CRISPR strategy) to knockout target-genes. Moreover, through this simple approach it is possible to "*fill two needs with one deed*", which means that we may both overcome blast disease menace to rice yield, and also generates a final bioproduct (commercial resistant cultivar) that tends to bypass GMO *tangled* regulation.

In this context, we have chosen to combine the agriculture needs of more basic studies in the rice-*M. oryzae* interaction, that ultimately holds the potential to contribute with discoveries on desirable new sources of rice resistance, with a CRISPR/Cas fitting strategy to the knockout of rice genes associated with blast susceptibility.

To do so, we first aimed to portray the foundation of rice susceptibility responses to blast disease, using a shotgun proteomics approach to question the proteome profiles between two near-isogenic lines, displaying contrasting phenotypes upon fungus infection. Bearing in mind the *S*-gene concept [1] and thus its potential application to build rice resistance, we focused our efforts on the identified proteins that showed a notable contrast in abundance between the proposed interactions, particularly those up-regulated at early time point (12 hpi) of infection in the susceptible condition, highlighted in the chapter II (figures 2 and 3 - pages 5 and 6; figure S2-B – page 13). According to the biological functions assigned to a specific set of differentially-abundant proteins (DAPs) revealed in the study, considering also their records from literature, we hypothesized about the fungus ability in hamper rice resistance by modulating specific host targets engaged (direct or indirect) with disease susceptibility.

For example, the identification of a sucrose synthase-like enzyme, 3.75 (log2)-fold increased in the susceptible interaction at 12 hpi, suggested to us that *M. oryzae* somehow co-opt host metabolism to obtain nutritional advantages, ultimately, highly demanded by its intense

intracellular multiplication [2]. Moreover, there is mounting evidence showing that most host-adapted pathogens rely on the activation and/or upregulation of certain plant genes (known as susceptibility S-genes) to meet their metabolic (carbon and other nutrients) requirement for proliferation and spread. In addition, aligned with our results, a notable enhanced expression and activity of plant invertase enzymes associated with fungus compatible interaction has been reported by several previous studies [3, 4].

A well-known characteristic observed in plant-pathogen incompatible interaction is the rapid mounting activation of host defense mechanisms, in which the oxidative burst of reactive oxygen species (ROS) plays key roles in resistance outcome, activating plant immune responses [5]. Hence, a set of differentially-abundant proteins composed by OsPIN1 (7.02 [log2]-FC), OsSRP-ZXA (4.07 [log2]-FC), OsNDX15 (4.02 [log2]-FC), and OsDjA2 (3.58 [log2]-FC, identified in the susceptible/resistant comparison at 12 hpi, deserved our attention as it enclosed proteins showing antioxidant activities, responsible for the maintenance of cellular redox homeostasis during stress. According to literature, diverse antioxidant-related proteins have been argued to abrogates a robust stimulation of host stress responses upon *M. oryzae* infection, leading to disease [6-11]. Moreover, a deeper analysis of our data revealed a set composed by eighteen cell-protective antioxidant ROS-scavenger enzymes, including peroxidase showing glutaredoxin/thioredoxin activities, was identified at the early stage of infection, suggesting here to be modulated by the fungus to 'keep a calm environment' for successful colonization. We proposed also a model for the regulation of rice susceptibility by *M. oryzae*, that captures the most relevant DAPs and their suggested roles upon infection (chapter II, figure 5 – page 9).

Taken together, our findings suggested a cause-effect relationship between the negative regulation of PTI/ETI defense-related pathways, triggered mostly by fungus modulation of the host cell antioxidant machinery, and the plant susceptibility outcome.

To test the hypothesis about the hijack of rice plant responses by the fungus *M.* oryzae, we considered to functionally validate our targets using antisense technology. The objective here was to query a functional relationship between the positive regulation of potential rice *S*-gene candidates and blast susceptibility outcome. Firstly, we carried out a gene expression analysis via qRT-PCR (chapter II, figure 4 – page 7) that showed an overall positive correlation at transcript level for most of tested candidates, which in some degree strengthened our selection criteria to pick the better targets for gene silencing. To reinforce our set of *S*-gene candidates, as well as to broaden the frame of prospection, we went through transcriptomics results generated in a previous study [12]. We ended up with three targets to the functional assay, *OsDjA2* (LOC_Os02g56040) from proteomics, which showed a remarkably increased abundance (3.58 [log2]-fold change) in the susceptible interaction at 12 h post infection with

M. oryzae (Chapter II, Table S1_spreadsheet 2 – data under request); *OsERF104* (LOC_Os08g36920) and *OsPYL5* (LOC_Os05g39580), the most notable differentially-expressed genes (DEG) (4.22 and 4.97 [log2]-fold change, respectively) identified in transcriptomics analysis 24 h post infection with *M. oryzae* in the same susceptible interaction.

Finally, we employed a transient-inducible gene silencing (TIGS) *in planta* assay to characterized their function in rice susceptibility. TIGS consists of a high-throughput system applied for transient knock-down (post-transcriptional regulation) of plant gene expression [13], that uses antisense DNA oligonucleotides to modulate targeted transcripts. Our results showed that rice plants pre-treated with phosphorothioate (PS)-modified DNA antisense oligos (PTO) targeting transcripts of our candidate *S*-genes displayed significant decrease in foliar blast disease symptoms upon infection, when compared with both the no-target control PTO and non-infected control plants (Chapter II, figure 6 – page 10). Our findings are widely supported by similar relevant results in literature. For example, in a proof-of-concept study using antisense phosphorothioate (PS)-oligodeoxynucleotides, Dinç E. et al. [14] showed that mRNA and protein levels in infiltrated leaves of *A. thaliana* were significantly reduced by up to 85% and 72%, respectively, in comparison with non-treated leaves. In a very recent study, Lambertucci et al., 2019 [15] used PTO-based TIGS *in planta* assay to modulate the transcript levels of the gene *TLP5* (thaumatin-like protein), highly induced in barley (*Hordeum vulgare*) leaves upon fungus infection. Authors demonstrated not only the efficacy of PTOs in modulate the targeted transcripts, confirmed via qRT-PCR analysis, but also revealed the engagement of *TLP5* with plant susceptibility as silenced infected plants showed an increased ROS burst that contributed to disease resistance. In addition, their strategy of placing the phosphorothioate (PS)-chemical tags in the oligo, which directly impacts on oligo intracellular stability, were exactly the same that we used to design our oligos.

In view of our results, we successfully employed shotgun proteomics and antisense-based gene silencing to prospect and functionally validate rice potential susceptibility factors. Hence, although the molecular mechanism of rice susceptibility to *M. oryzae* is far from being fully captured [16], we suggested that a rational manipulation of host susceptibility genes represents an effective strategy to manage blast disease, serving as an alternative strategy to *R* genes in breeding programs, and a flourishing approach to build rice resistance via new breeding techniques.

Since physiological expression of *S*-genes may impact plant resistance during infection [17], it represents a very suited target to CRISPR/Cas9 knockout strategy. Nevertheless, although several recent studies using RNAi gene silencing technology and/or overexpression have revealed a series of *S*-genes in different pathosystems, including rice-*M. oryzae*, up to our

knowledge there are very few studies that used the present *S*-gene knockout strategy via CRISPR/Cas9 to enhance rice resistance to blast disease.

Here, we aimed to knockout by CRISPR/Cas9 system the two rice genes *OsDjA2* and *OsERF104*, encoding a chaperone protein and an APETELA2/ethylene responsive factor, respectively, which showed the best results in our previous functional validation assay, and were suggested as a potential rice *S*-genes. Our choice of candidate targets for genome editing was also supported by literature findings. OsDjA2, also known as DnaJ, Hsp40, and J-proteins, consists in a "Heat Shock Protein" (Hsp), implicated in a myriad of functions in diverse plant species, with essential and regulatory role in plant innate immune response [18]. Regarding its function during rice-*M. oryzae* pathosystem, consecutive studies have succeeded to functionally characterize the role of *DnaJ* homologues rice genes in blast susceptibility. A seminal study was carried out by Zhong and colleagues [19] in which was observed the increased expression of rice DnaJ gene during a compatible interaction with the fungus *M. oryzae*. Using RNA interference (RNAi) technology, authors reported the role of chaperone DnaJ protein, OsDjA6, in the negative modulation of rice basal resistance upon *M. oryzae* infection. They observed that RNAi *OsDjA6* mutant plants exhibited increased levels of reactive oxygen species (ROS) burst accumulation as well as up-regulation of defense-related genes, hence, enhanced resistance to *M. oryzae* in comparison with wild-type plants. Moreover, we have also few pieces of evidences that in viral pathogenesis, for example, the silencing of diverse J-domain-containing protein can lead to resistance or susceptible outcomes [20]. Our second candidate target, OsERF104, member of APETALA2/Ethylene Responsive Factor (AP2/ERF) gene family, argued to be involved in the tangled modulation of plant immunity. In response to phytohormones and biotic/abiotic stresses, members of AP2/ERF gene family can positively or negatively regulate plant resistance [21]. Further, increasing evidence indicates that AP2/ERF TF may act as molecular switches to regulate clusters of stimuli responsive genes, playing key roles in negative regulation of ET biosynthesis and rice susceptibility during distinctive plant-fungal interactions. For example, recent investigations have reported different plant-specific ERF genes acting by suppressing PAMP-triggered immunity (PTI) upon pathogen infection. Wenqin Lu et al. [22] revealed the importance of *AtERF019* gene 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 [23]. Regarding rice-M. oryzae pathosystem, Dongfeng and co-workers [24] 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 promptly increase in the expression of pathogenesis-related (PR) gene products. Later, a pioneer study was

performed by Wang and coworkers [25] where they achieved to enhance rice blast resistance by CRISPR/Cas9 knockout of the same ERF transcription factor gene *OsERF922*, asserting the role of this AP2/ERF domain-containing gene in rice-blast susceptibility.

To knockout our target-genes, we firstly designed each sgRNAs and cloned into a simplex CRISPR vectors, kindly provided by C. Périn, from CIRAD, UMR AGAP, France (Chapter III, figure 1 – page 94). Next, rice plants (cv. Nipponbare) were transformed via coculture with *A. tumefaciens* containing the T-DNA vectors harboring only one sgRNA, at a time. Hygromycin-containing medium was used to select all resistant rice calli that were subsequently regenerated as potentially transformed (edited) plants. The molecular characterization analysis of InDel-induced mutation impacts at the gene and protein levels for both targeted-gene revealed different nucleotide editing forms, mostly of them inducing loss-of-function mutation types (Chapter III, figure 1 – page 96; table 1 – pages 98 and 99; figure S1-S3 – pages 114 to 116). Finally, knockout mutant lines for each targeted gene were tested for disease resistance. The phenotyping analysis revealed not only a significant decrease in the number of blast lesions, but also a reduction in the percentage of lesioned leaf area when compared with non-edited control lines (Chapter III, figure 4 – page 103).

Therefore, our results not only corroborate our previous findings that suggested the ability of 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, but also plead CRISPR/Cas9 knockout of rice susceptibility genes as an effective and alternative strategy that should be better exploited towards a more resolute resistance to blast disease.

Conclusions and perspectives

The central goal of the thesis was to depict the basis of rice-blast susceptibility, focusing on the quota of *S*-gene contribution in it. In the part related to the prospection of those players, our approach using shotgun comparative proteomics enabled us to provided important molecular insights on rice-*M. oryzae* pathosystem at early stages of infection, disclosing potential rice susceptibility factors with pivotal and specific roles in triggering a pathogen compatible state. Precisely in the functional validation part, our choice to use a relative new strategy, at least in plants, to gene silencing of our most prominent targets, not only guided us to select the most suited genes to the downstream analysis, but also shed light on such innovative approach to transiently modulate plant transcripts of interesting. Unlike RNAi technology, PTO-based TIGS does not requires the time-consuming steps of vector assembling, genome integration and expression to operate the target knockdown. In our last move, the

application of CRISPR/Cas9 system to delete a couple of rice *S*-genes demonstrated the suitability of the alternative strategy toward the development of genetically improved rice crops against the hemibiotrophic fungus *M. oryzae*. Furthermore, we have already designed new CRISPR vectors, simplex and multiplex (combining more than one sgRNA), to transform elite rice varieties aiming to generate new commercial breeding products.

Taken together, due to the contribution of all functional genomic approaches, our findings provide novel tools to deal with *M. oryzae*, the major threat for rice paddies, and may be also exploited in different pathosystems, leading to relevant contributions in the fight for global food security.

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