Universitat Autònoma de Barcelona

Understanding the role of iron homeostasis in rice immunity and novel applications of miRNAs for crop protection

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Ph. D. Thesis

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Dissertation presented by Ferran Sánchez Sanuy for the degree of Doctor of Biology and Plant Biotechnology by the *Universitat Autònoma de Barcelona*.

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Outline of the Thesis

This Ph. D. Thesis is organized into chapters according to subject matter (Chapters I to III).

The **General Introduction** describes aspects related to the model system used in this research, the interaction of rice plants with the fungal pathogen *Magnaporthe oryzae*. This fungus is the causal agent of the rice blast disease. I summarize current knowledge on mechanisms in plant immunity, with special emphasis on connections between iron homeostasis and plant immunity. Afterwards, I present current knowledge on microRNAs (miRNAs), including miRNAs involved in plant immunity and nutrient homeostasis, cross-kingdom movement of small RNAs, and future prospects on miRNA-based strategies for crop protection

In **Chapter I,** I describe the involvement of miR7695 in the regulation of gene expression during infection of rice plants with the fungus *M. oryzae*. MiR7695 targets *OsNramp6* (*Natural resistance-associated macrophage protein 6*) encoding an iron transporter from rice. Results obtained in this chapter were published (**Sánchez-Sanuy *et al.* 2019**. BMC Plant Biology). Related with the topic in this chapter, early during my Ph. D. Thesis, I participated in studies that revealed the relevance of iron supply on blast resistance (**Peris-Peris *et al.* 2017**. Molecular Plant-Microbe Interaction).

In **Chapter II**, I examined the sites of iron accumulation, and the transcriptional responses of rice leaves to iron treatment, in the context of blast resistance. Iron accumulation and ROS production were found to accumulate at the sites of fungal penetrations in rice plants grown under high Fe supply, these plants also exhibiting blast resistance. Iron supply promotes the super-activation of defense-related genes and phytoalexin

accumulation. Results obtained in this study are being prepared for publication.

In **Chapter III,** I explored the feasibility of using tailor-made miRNAs as antifungal agents protecting rice from the blast fungus by topical applications. The rationale behind this study was to demonstrate the capability of miRNAs/miRNA precursors to silence *M. oryzae* genes relevant for pathogen virulence (cross-kingdom RNA interference). Two target genes were selected for this study, *MoPMK1* (mitogen-activated protein kinase) and *MoMAC1* (adenylate cyclase). Three different approaches were tested based on the use of: (i) chemically-synthesized mature miRNAs, (ii) *in vitro*-synthesized precursor miRNAs and (iii) *in vivo*-synthesized precursor/mature miRNAs (in the heterologous system of *Nicotiana benthamiana* leaves). I demonstrate that the exogenous application of miRNAs directed against *MoPMK1* is effective to reduce blast disease symptoms in rice leaves. Results obtained in this study are being prepared for publication.

In the **General Discussion** I summarize key findings from the three chapters and discuss key findings of my research and implications for protection of rice plants against the fungus *M. oryzae*.

During the course of my Ph. D. Thesis, I also contributed to other research activities going on in the group. Specifically, I worked in the functional characterization of miR812w, a new member of the miR812 family of rice, that plays a role in blast resistance by directing DNA methylation *in trans* at target genes as well as in *cis* at the MIR812w locus. Results obtained in this study were published (**Campo *et al.* 2021**. Plant Biotechnology Journal 2021).

Published papers (Annex I to III) are availabe online:

Annex I. Sánchez-Sanuy F, Peris-Peris C, Tomiyama S, Okada, K, Hsing YI, San Segundo B, Campo S. "*Osa-miR7695 enhances transcriptional priming in defence responses against the rice blast fungus*". BMC Plant Biology. 2019; 19:563.

Annex II. Peris-Peris C, Serra-Cardona A, **Sanchez-Sanuy F**, Campo S, Ariño J, San Segundo B. "*Two NRAMP6 isoforms function as iron and manganese transporters and contribute to disease resistance in rice*". Molecular Plant Microbe Interactions. 2017; 30:385–398.

Annex III. Campo S, **Sánchez-Sanuy F**, Camargo-Ramírez R, Gómez-Ariza J, Baldrich P, Campos-Soriano L, Soto-Suárez M, San Segundo B. "*A novel transposable element-derived microRNA participates in plant immunity to rice blast disease*". Plant Biotechnology Journal. 2021; 19:1798–1811.

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Summary

Plants are constantly challenged with a wide range of environmental stresses that cause major losses in crops. These stresses are categorized into biotic or abiotic stresses depending on their nature. Biotic stresses are caused by living organisms like fungi, oomycetes, bacteria, viruses, nematodes, insects and weeds. Abiotic stresses include salinity, drought, extreme temperatures, flooding, radiation, as well as nutrient stress or heavy metals toxicity, among others. Due to their sessile lifestyle, plants cannot escape from stresses and have evolved a set of mechanisms to overcome them. To cope with environmental stress, plants are able to sense the stimuli for the activation of signal transduction pathways leading to transcriptional reprogramming of gene expression. Evidence supports that plants respond to multiple stresses differently from how they do to individual stresses, by activating a specific programme of gene expression depending on the type of stress encountered. For instance, the presence of an abiotic stress can have the effect of reducing or enhancing susceptibility to a pathogen, or *vice versa*. However, most studies on plant responses to biotic and abiotic stresses have been approached on plants subjected to an individual stress, but in nature they are more likely to occur simultaneously.

In plants, microRNAs (miRNAs) are key regulators of gene expression in diverse developmental processes and adaptation to environmental stress. Distinct miRNAs have been shown to be involved in the plant response to pathogen infection or nutrient homeostasis. Although a connection between iron and plant immunity has long been recognized in agronomy, the exact role of iron homeostasis during the plant defense response to pathogen infection is not well understood. In this Ph. D. Thesis, I investigated miRNA-mediated regulation in processes associated to iron (Fe) homeostasis and innate immunity in rice plants during infection with the rice blast fungus *Magnaporthe oryzae*.

On the other hand, it is well recognized that small RNAs are able to move between interacting organisms for cross-kingdom regulation of gene expression through RNA interference (RNAi). Recent evidence also

supports bidirectional cross-kingdom communication of small RNAs between host plants and adapted fungal pathogens that determine the outcome of infection. Most of these studies focused on the movement of long double stranded RNAs (dsRNAs) or small interfering RNAs (siRNAs). Moreover, the application of dsRNAs or siRNAs has proven to be effective for the control of fungal diseases. At present, little information is available about movement of miRNAs between organisms. As a first step to evaluate whether miRNAs can be useful tools for crop protection, in this Ph. D., I explored the feasibility of using tailor-made miRNAs to repress the expression of fungal genes during infection of rice plants with *M. oryzae*.

Resum

Les plantes s'enfronten constantment a una àmplia gamma d'estressos ambientals que provoquen importants pèrdues en els cultius. Aquests estressos es classifiquen, segons la seva naturalesa, en estressos biòtics o abiòtics. L'estrès biòtic és causat per organismes vius com fongs, oomicets, bacteris, virus, nematodes, insectes; o bé per altres plantes. Els estressos abiòtics inclouen fenòmens diversos com salinitat, sequera, temperatures extremes, inundacions, radiació; així com estrès nutricional o toxicitat per metalls pesants, entre d'altres. A causa del seu estil de vida sèssil, les plantes no poden escapar d'aquests estressos i han desenvolupat un conjunt de mecanismes per superar-los. Per fer front a l'estrès ambiental, les plantes poden percebre els estímuls i activar vies de transducció de senyals que condueixen a la reprogramació transcripcional de l'expressió gènica. Les evidències confirmen que les plantes responen a estressos múltiples de manera diferent a com ho fan a estressos individuals, activant un programa específic d'expressió gènica segons el tipus d'estrès trobat. Per exemple, la presència d'un estrès abiòtic pot reduir o augmentar la susceptibilitat a un patogen, o viceversa. No obstant això, la majoria dels estudis sobre les respostes de les plantes a l'estrès biòtic i abiòtic s'han realitzat en plantes sotmeses a un estrès individual, però en la naturalesa és més probable que hagin d'enfrontar-se a estressos múltiples.

En les plantes, els microARN (miARN) són reguladors clau de l'expressió gènica en diversos processos de desenvolupament i adaptació a l'estrès ambiental. S'ha demostrat que diferents miARNs participen en la resposta de la planta a la infecció per patògens o l'homeòstasi de nutrients. Encara que des de fa molt temps en l'agronomia s'ha reconegut una connexió entre el ferro i la immunitat de les plantes, no es comprèn bé el paper exacte de l'homeòstasi del ferro durant la resposta de defensa de les plantes a la infecció per patògens. En aquesta tesi doctoral, he investigat la regulació de processos associats a l'homeòstasi del ferro (Fe) regulada per miARN i la immunitat innata en plantes d'arròs durant la infecció amb el fong de la piricul ariosi de l'arròs *Magnaporthe oryzae*.

D'altra banda, se sap que els petits ARN es poden moure entre organismes que interactuen per a la regulació de l'expressió gènica entre regnes mitjançant la interferència d'ARN (ARNi). L'evidència recent també dona suport a la comunicació bidireccional entre regnes de petits ARN entre plantes hospedants i patògens fúngics adaptats que determinen el resultat de la infecció. La majoria d'aquests estudis es centren en el moviment d'ARNs bicatenaris llargs (dsRNAs) o petits ARN interferents (siARNs). A més, l'aplicació de dsARNs o siARNs ha demostrat ser eficaç per al control de malalties fúngiques. En l'actualitat, es disposa de poca informació sobre el moviment de miARNs entre organismes. Com a primer pas per avaluar si els miARNs poden ser eines útils per a la protecció de cultius, en aquesta tesi he explorat la viabilitat d'utilizar miARNs fets a mida per reprimir l'expressió de gens fúngics durant la infecció de plantes d'arròs amb *M. oryzae*.

Resumen

Las plantas se enfrentan constantemente a una amplia gama de estreses ambientales que provocan importantes pérdidas en los cultivos. Estos estreses se clasifican, según su naturaleza, en estreses bióticos o abióticos. El estrés biótico es causado por organismos vivos como hongos, oomicetos, bacterias, virus, nematodos, insectos; o bien por otras plantas. Los estreses abióticos incluyen fenómenos diversos como salinidad, sequía, temperaturas extremas, inundaciones, radiación; así como estrés nutricional o toxicidad por metales pesados, entre otros. Debido a su estilo de vida sésil, las plantas no pueden escapar de estos estreses y han desarrollado un conjunto de mecanismos para superarlos. Para hacer frente al estrés ambiental, las plantas pueden percibir los estímulos y activar vías de transducción de señales que conducen a la reprogramación transcripcional de la expresión génica. Las evidencias respaldan que las plantas responden a estreses múltiples de manera diferente a como lo hacen a estreses individuales, activando un programa específico de expresión génica según el tipo de estrés encontrado. Por ejemplo, la presencia de un estrés abiótico puede reducir o aumentar la susceptibilidad a un patógeno, o viceversa. Sin embargo, la mayoría de los estudios sobre las respuestas de las plantas al estrés biótico y abiótico se han realizado en plantas sometidas a un estrés individual, pero en la naturaleza es más probable que deban enfrentarse a estreses múltiples.

En las plantas, los microARNs (miARNs) son reguladores clave de la expresión génica en diversos procesos de desarrollo y adaptación al estrés ambiental. Se ha demostrado que distintos miARNs participan en la respuesta de la planta a la infección por patógenos o la homeostasis de nutrientes. Aunque desde hace mucho tiempo en la agronomía se ha reconocido una conexión entre el hierro y la inmunidad de las plantas, no se comprende bien el papel exacto de la homeostasis del hierro durante la respuesta de defensa de las plantas a la infección por patógenos. En esta tesis doctoral, he investigado la regulación mediada por miARNs en procesos asociados a la homeostasis del hierro (Fe) y la inmunidad innata

en plantas de arroz durante la infección con el hongo del añublo del arroz *Magnaporthe oryzae*.

Por otro lado, es bien sabido que los pequeños ARN pueden moverse entre organismos que interactúan para la regulación de la expresión génica entre reinos a través de la interferencia de ARN (ARNi). La evidencia reciente también respalda la comunicación bidireccional entre reinos de pequeños ARNs entre plantas hospedantes y patógenos fúngicos adaptados que determinan el resultado de la infección. La mayoría de estos estudios se centran en el movimiento de ARN bicatenarios largos (dsARNs) o pequeños ARN interferentes (siARNs). Además, la aplicación de dsARNs o siARNs ha demostrado ser eficaz para el control de enfermedades fúngicas. En la actualidad, se dispone de poca información sobre el movimiento de miARNs entre organismos. Como primer paso para evaluar si los miARNs pueden ser herramientas útiles para la protección de cultivos, en esta tesis he explorado la viabilidad de usar miARNs hechos a medida para reprimir la expresión de genes fúngicos durante la infección de plantas de arroz con *M. oryzae*.

General introduction

1. Rice

1.1 Agronomic importance of rice

Rice, wheat, and maize are the world's three leading *food* crops (FAO, 2021). Rice is the staple food of more than 3.5 billion people around the world (half of the world's population) with greater importance for developing countries in terms of food security and alleviation of malnutrition. Rice cultivation reaches more than 150 million hectares globally (about the 10% of arable land) and 90% of the world's rice is produced and consumed in Asia, being China and India, the major producers **(Figure 1)** (FAO, 2021). In Europe, Italy is the major rice producer, followed by Spain, France and Greece (FAO 2021; McLean *et al.* 2013). The actual increase of the world population is forecast to reach 9 billion people by 2050. Thus, it is necessary to increase global crop yields to satisfy the expected demand.

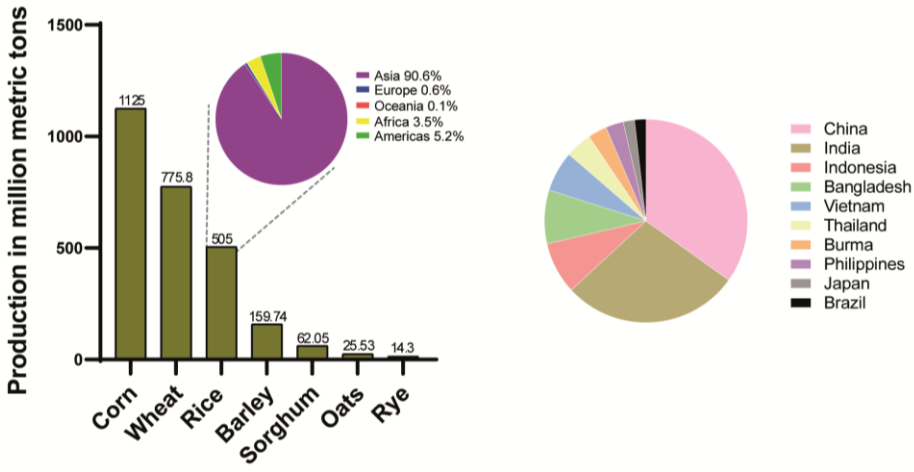

Figure 1. Worldwide grain production in million metric tons in 2020/21 (left panel). Top 10 rice producing countries (right panel). Source: FAOSTAT stats 2020/21.

Rice originated around 9.000 years ago in the Yangtze Valley (China) and has a long history of natural selection and domestication (Gutaker *et al.* 2020). Rice can be grown in a wide range of environments. Irrigated systems cover more than half of the world's rice lands and it produces 75% of the global rice supply, although a high demand of water is implied. Rice cultivation is threatened by biotic and abiotic stresses that negatively affect rice production. One of the major factors limiting rice production is the occurrence of diseases caused by various fungal, bacterial and viral pathogens. The most devastating fungal disease affecting rice production is rice blast, caused by the fungal pathogen *Magnaporthe oryzae* (Dean *et al.* 2012; Wilson and Talbot 2009).

1.2 The rice plant

Rice is a flowering plant included in the Liliopsida class (or Monocotyledonae), pertaining to the family of grasses (Poaceae or Gramineae) within the *Oryza* botanical genus. Rice has a relatively small compact genome, the smallest among all cereal crops, of about 430 Mbp in 12 chromosomes. Within the genus *Oryza*, different types of genomes can be found, both diploid (AA, BB, CC, EE, FF and GG; 2n=24) and tetraploid (BBCC, CCDD, KKLL and HHJJ; 4n=48). The *Oryza* genus contains 23 different species, of which only two are cultivated: *Oryza sativa* ('Asian rice') and *O. glaberrima* ('African rice') which were independently domesticated in Asia and Africa. *O. sativa* domestication took place 9.000 years ago from *O. rufipogon* (perennial) and *O. nivara* (annual) and comprises two main types, *indica* and *japonica*. *O. sativa* spp. *japonica* is further differentiated into *temperate japonica* (*japonica*) and *tropical japonica* (*javanica*) varieties (Garris *et al.* 2005). The *indica* type has long, wide to narrow, light green leaves, profuse tillering, usually long and thin grains and secondary ramifications (small branches) in the panicle holding the grains. The *japonica* type is characterized by thin, light green leaves, medium tillering, short to intermediate size, and rather

short and round grains (http://www.ricehub.org/). *O. glaberrima* originated in the inland delta of the Niger River and show profuse vegetative development, few secondary ramifications and red caryopsis (a grain with a red pericarp). Nowadays, the Asian species (*O. sativa*) are cultivated far more than the African species (*O. glaberrima*), because of its higher yield potential. The rice wild species show high genetic diversity offering a reservoir for agronomic traits of interest (e.g. seed characteristics or resistance genes, among others). Wild species include *O. officinalis*, *O. punctata*, *O. barthii*, *O. rhizomatis*, *O. rufipogon*, *O. nivara*, *O. meridionalis*, *O. granulate*, among others. **Figure 2** shows morphological traits of cultivated and wild species (Linares 2002; Londo *et al.* 2006).

Figure 2. Representative species of *Oryza*. Adapted from Zhang and Wing, 2013.

Rice plants take around 4–6 months to grow from seeds to mature plants, depending on the variety and environmental conditions. They undergo three general growth phases: vegetative, reproductive, and ripening (grain filling) **(Figure 3)**. Reproductive stage (panicle initiation to flowering) and ripening stage (flowering to harvesting) is constant at about 1 month, each. Only vegetative growth stage (sowing to panicle initiation) is variable and depends on variety (https://ricepedia.org/). Tillering starts at 2-3 weeks after sowing.

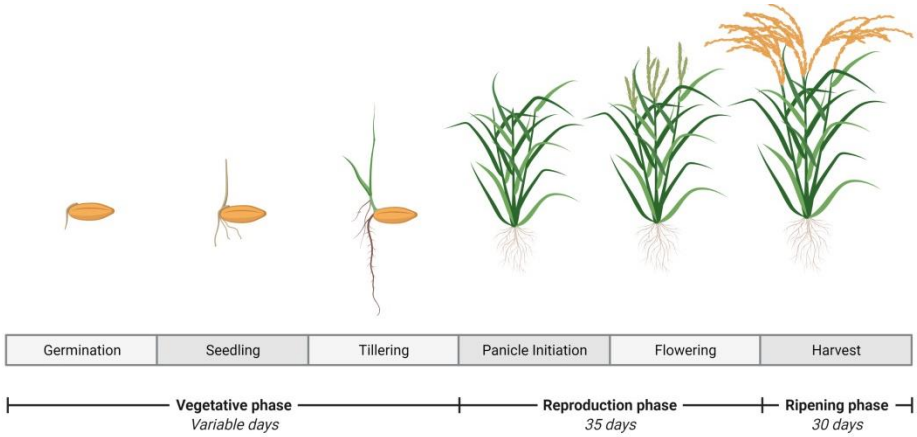

Figure 3. The rice growth stages.

The rice anatomy is shown in **Figure 4**. Rice has fibrous roots covered with root hairs, with a primary root and various secondary roots. Like other Gramineae, the root system of rice is relatively shallow. The stem is composed of a series of nodes and internodes, and each node has one leaf **(Figure 4)**. The stem's main function is to transport water and nutrients and to bring air to the roots. The sheath is the leaf part that wraps up the nodes/internodes of the stem at the leaf base. The junction point between the leaf and the steam sheath is called the collar and two elements can be found: the auricle and the ligula **(Figure 4)**. Panicles are composed of primary ramifications (small branches) with secondary branches carrying the spikelets. One single panicle can bear between 50 and 500 spikelets.

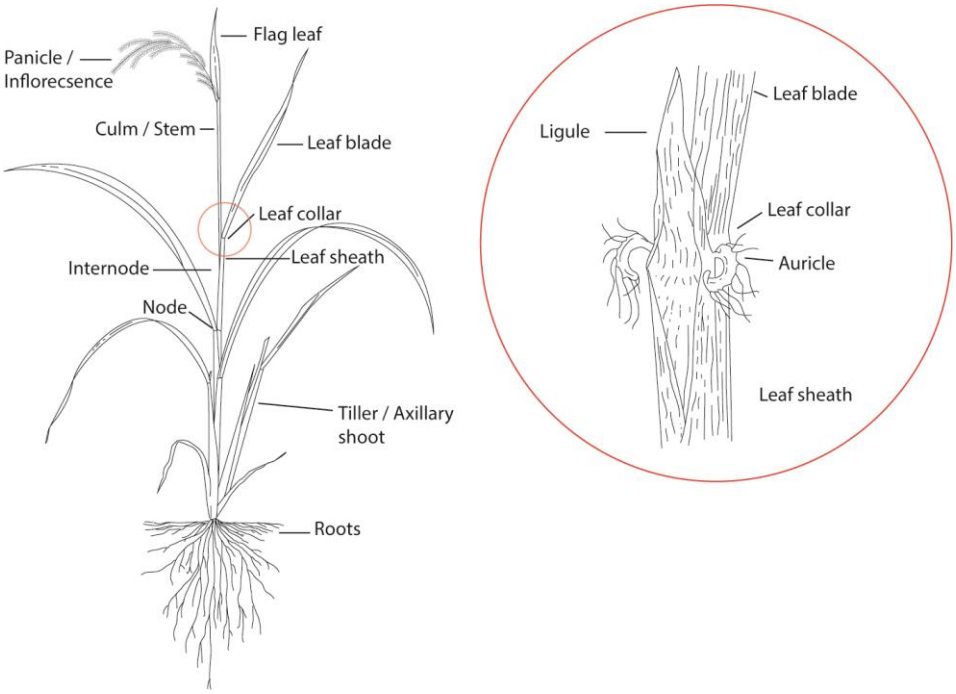

Figure 4. The rice plant anatomy.

1.3 Rice as a model for cereal research

Rice is the *model* system for functional genomics studies in monocotyledonous plants (cereals). High-quality reference genomes are available for *O. sativa japonica* and *indica* subspecies (Goff *et al.* 2002; Li *et al.* 2014). The project "3,000 rice genomes" (3.000 Rice Genomes Project, 3K RGP), carried out in collaboration by researchers from the Chinese Academy of Agricultural Sciences (CAAS), the Institute International Rice Research (IRRI) and the Beijing Genomics Institute (BGI), provided the genome sequence of 3.024 rice varieties from 89 countries (Li *et al.* 2014). High resolution genome-wide association mapping platforms have been launched (McCouch *et al.* 2016). Large collections of expressed sequence tag (EST), insertion mutants (T-DNA) and transposon mutants are publicly available (Lo *et al.* 2016; Ram *et al.*

2019; Wang *et al.* 2013). Efficient rice transformation protocols have been developed, including *Agrobacterium*-mediated transformation that allow transgene expression or genome editing modifications (Sallaud *et al.* 2003). Genome editing technologies are based on the creation of double-stranded breaks at target genome sequences by TALENs (Transcription activator-Like Effector Nucleases) or CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats) systems (Lowder *et al.* 2015). Efficient genome editing has been achieved in rice plants using the CRISPR/Cas9 system (Miao *et al.* 2013). CRISPR has expanded the range of possible genome editing modifications with high specific targeting (Hu *et al.* 2016; Lowder *et al.* 2015). Thus, in addition to its agronomic and economic relevance, rice represents a good model for basic research in cereals.

2. Plant pathogens

Plant pathogens have been a huge problem affecting crops worldwide with economic and social implications. In addition, globalization has made the world completely connected, facilitating the introduction of non-indigenous plant pathogens into new environments (Santini *et al.* 2018). This lack of plant-pathogen environmental coevolution leads to potentially encounter new host species lacking resistance mechanisms, creating favorable new environments for pathogen development and huge implications in plant-based production systems. Pathogens can cause losses in yield up to 100% but they can also reduce quality. Plant pathogens belong to different domains of life, including viruses, bacteria, fungi, oomycetes and parasitic plants. The most important biological constraint caused by plant pathogens occurred the late 1840s in Ireland by *Phytophthora infestans*, an oomycete which caused the Irish potato famine diminishing Irish population up to 25%.

Plant pathogens can be classified as avirulent and virulent pathogens. Avirulent pathogens are those that do not cause measurable damage to the host (incompatible interactions), whereas virulent pathogens cause measurable damage to the host, and disease (compatible interactions). Moreover, the developmental and/or nutritional status of the plant, as well as other environmental factors can affect the outcome of a given plant-pathogen interaction (Velásquez *et al.* 2018). Depending on their lifestyle, plant pathogens can be classified as biotrophic, necrotrophic or hemibiotrophic pathogens (Geeta and Mishra 2018). Biotrophic pathogens are those that establish a feeding relationship with the living cells of their hosts, rather than killing the host cells. Examples of biotrophic fungal pathogens greatly affecting agricultural production are those causing the Powdery mildew disease affecting a wide range of plant species, monocotyledonous and dicotyledonous species. In monocots, *Blumeria graminis hordei* and *B. graminis triti* infect barley and wheat, respectively (Dean *et al.* 2012). Necrotrophic pathogens kill invaded cells resulting in extensive necrosis, tissue maceration, and plant rots. *Botrytis cinerea* is a necrotrophic pathogen that causes devastating diseases and significant crop losses affecting several plants hosts (Cheung *et al.* 2020). Hemibiotrophic fungi present a first stage as biotrophs to then switch to a nectrotrophic lifestyle.

2.1 Major pathogens of rice

Rice production is threatened by the presence of various pathogenic microorganisms (fungi, bacteria and viruses) as well as by insects, nematodes and weeds. It is estimated that 20% - 40% of the worldwide production in rice is lost due to diseases (http://www.fao.org/; FAO, 2020). Yield losses can vary depending on environmental conditions that can favor or harm pathogen development, and fluctuate between seasons and regions. When conditions are favorable for the pathogen, reductions of up to the 100% of the yield can occur in susceptible varieties. The International Rice Research Institute (IRRI)

promotes the use of resistant varieties in order to reduce the impact of rice diseases.

Regarding fungal pathogens, *M. oryzae* is the most devastating fungal pathogen of rice, being responsible of important losses in rice production worldwide (discussed in section 2.2). Rice is also affected by other fungal pathogens, such as *Rhizoctonia solani*, *Gibberella fujikuroi*, causing sheath blight and bakanae, respectively (Singh *et al.* 2019; Singh and Sunder 2012). Major rice diseases and causal agents are shown in **Table 1**.

Bakanae ("foolish seedling" in Japanese) disease, caused by one or more seed-borne *Fusarium* species, mainly *F. fujikuroi*, is a disease of increasing economic importance in major rice-producing areas (Singh and Sunder 2012). The fungus infects rice plants from the seedling stage to the mature stage, with severe infection of rice seeds. Symptoms of the disease are poor seedling emergence, abnormal elongation of internodes in the seedlings due to the ability of *F. fujikuroi* to produce gibberellic acids (GAs), and grain contamination (Cen *et al.* 2020). *Fusarium* species also produce mycotoxins in rice grains that represent serious food safety concerns in animal and human health (Munkvold 2017).

Regarding bacterial pathogens, bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (also referred to as *Xoo*) is the most important bacterial disease in rice (Niño-Liu and Bogdanove 2006). Persistent in warm humid areas, bacterial blight has been documented in Asia, the western coast of Africa, Australia, Latin America, and the Caribbean (EFSA Panel on plant health, 2018). Bacterial blight causes wilting of seedlings and yellowing and drying of leaves. Bacteria can travel through the water from infected plants and colonize the roots of neighbor healthy plants. Other bacterial diseases are caused by *X. oryzae* pv. *oryzicola*, responsible of the bacterial leaf streak and *Pseudomonas fuscovaginae* (sheath brown rot).

	Disease	Causal agent	Symptoms
Bacteria	Bacterial blight	*Xanthomonas oryzae pv. oryzae*	Wilting and yellowing of leaves
	Bacterial leaf streak	*Xanthomonas oryzae pv. oryzicola*	Browning and drying of leaves
	Bacterial sheath brown rot	*Pseudomonas fuscovaginae*	Rotting in sheaths and grains of seedlings and mature plants
Fungi	**Blast**	***Magnaporthe oryzae***	**White to gray-green lesions or spots**
	Brown spot	*Cochliobolus miyabeanus*	Big brown spots on the leaves
	False smut	*Ustilaginoidea virens*	Chalkiness of grains
	Sheath blight	*Rhizoctonia solani*	Leaves senesce or dry out
	Leaf Scald	*Microdochium oryzae*	Scalded appearance of leaves
	Narrow brown spot	*Sphaerulina oryzina*	Premature death of leaves and leaf sheaths, premature ripening of grains, lodging of plants
	Red stripe	*Gonatophragmium spp.*	Orange spot with a stripe
	Bakanae	*Gibberella fujikuroi*	Seedborne fungal disease. Abnormally tall, thin leaves, fewer tillers, empty grains
	Sheath rot	*Sarocladium oryzae*	Reduces grain yield (unfilled seeds and sterile panicles)
	Stem rot	*Sclerotium oryzae*	Lesions and production of chalky grains and unfilled panicles
Virus	Rice stripe virus disease	*Rice stripe virus*	Chlorotic white stripes, mottling, and necrotic streaks on the leaves
	Rice Yellow Mottle Virus	*Rice Yellow Mottle Virus*	Brown to dark-brown discoloration and poor panicle exsertion
	Rice grassy stunt	*Rice grassy stunt virus*	Severely stunted plants
	Rice ragged stunt	*Rice ragged stunt virus*	Unfilled grains and plant density loss
	Tungro	*Mixed infection rice tungro baciliform virus and rice tungro spherical virus*	Leaf discoloration, stunted growth, reduced tiller numbers and sterile or partly filled grains

Table 1. Major rice diseases. Source: IRRI.

2.2 *Magnaporthe oryzae*

Blast disease or Piriculariosis caused by the ascomycete filamentous fungus *Magnaporte oryzae* (anamorph *Pyricularia oryzae)* is the most devastating disease of cultivated rice worldwide (Dean *et al.* 2012; Wilson and Talbot 2009). *M. oryzae* infects more than 50 different grass species, some of them with relevant agronomic importance as rice, wheat, barley, oat, and millet (Langner *et al.* 2018). *M. oryzae* wheat blast first appeared in Brazil at 1985 and rapidly spread across South America (Cruz and Valent 2017). Later, wheat blast appeared in Bangladesh, the world's second largest wheat producer (Singh *et al.* 2021).

M. oryzae can infect leaves, leaf collars, necks and panicles of rice plants. This is an ascomycete fungus with a hemibiotrophic lifestyle that involves initial proliferation inside living host cells before switching to a destructive necrotrophic mode (Fernandez and Orth 2018;Wilson and Talbot 2009). The blast disease cycle starts when a conidium reaches the host tissue and develops a germ tube, which grows on the leaf surface **(Figure 5)**. The adherence of the conidium to the hydrophobic surface of the leaf occurs through an adhesive substance (spore-tip mucilage) (Wilson and Talbot 2009). A polarized germ tube develops from the conidium which culminates in the formation of a dome-shaped infection structure, or appressorium. Maturation of the appressorium is accompanied by rapid synthesis of glycerol, leading to turgor generation and formation of a thick differentiated melanin layer on the inner side of the appressorium cell wall (Wilson and Talbot 2009). This results in mechanical strength that facilitates the fungus entrance into the host tissue through leaf cuticle. A penetration peg develops from the appressorium to enter into the epidermal cells, where it expands to become an infective hypha (Howard and Valent 1996; Wilson and Talbot 2009). Invasive growth of *M. oryzae* involves a biotrophic stage in which the fungus grows inside plant cells (thin hyphae) surrounded by the plasma membrane of an invaginated cell. During the biotrophic stage, the hyphae move from cell to cell through plasmodesmata (Kankanala *et al.* 2007).

At this stage of the infection process, the fungus redirects nutrients from living plant cells to facilitate nutrient acquisition from the host tissue (Fernandez and Wilson 2012). Several days after infection, the fungus switches to a necrotrophic life style (thick bulbous hyphae) and blast lesions appear on the leaf surface, typically diamond-shaped lesions with a gray or white center and brown or reddish-brown border. In these lesions, the fungus sporulates allowing the disease to spread to neighboring plants. Favorable conditions for *M. oryzae* infection are a temperature of 25º - 28º C and high humidity. Other factors such as prolonged wetness and high nitrogen or phosphate application also favor the development of the disease (Ballini *et al.* 2013; Campos-Soriano *et al.* 2020). *M. oryzae* overwinters on crop debris, seeds and on weed hosts.

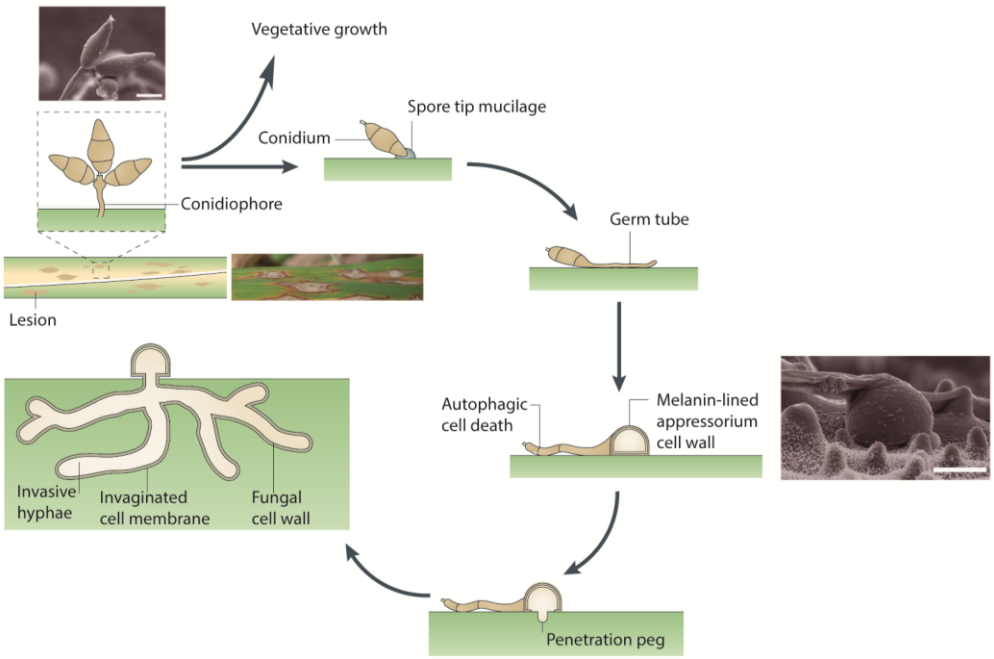

Figure 5. *Magnaporthe oryzae* life cycle. Adapted from Wilson and Talbot 2009.

The use of resistant varieties and chemical application are currently used to prevent *M. oryzae* prevalence (Upadhyay and Bhatta 2020). During the last years, special effort has been done to identify blast resistance genes and to generate rice resistant varieties by traditional breeding (Ning *et al.* 2020; Nishad *et al.* 2020; Srivastava *et al.* 2017; Wang *et al.* 2015). More than 100 genes and up to 350 traits loci have been identified as rice resistance genes (Li *et al.* 2019a; Monteiro and Nishimura 2018). However, traditional resistance conferred by *R* genes often breaks down in a few years due to the high variability and fast evolving fungal populations (Wu *et al.* 2019). Therefore, breeders usually prefer rice varieties that contain multiple R genes and have broad-spectrum blast resistance.

Treatment with systemic fungicides like triazoles and strobilurins has been used to prevent and control blast infection (Kongcharoen *et al.* 2020). But, abusive use of chemical compounds has arisen to resistant strains and concerned public health about their side-effects on non-target organisms and the environment. Chemical fungicides acting on the *M. oryzae* life cycle (e.g. inhibitors of melanin biosynthesis) are currently used for the control of the blast disease (Takagaki 2015). Chemical treatments aiming the activation of plant defenses, such as probenazole or spermidine, are also used for blast control.

Cultural methods aiming to create a less favorable environment for *M. oryzae* progression are also used to control *M. oryzae* incidence in the field (e.g. low plant density, precise nitrogen application or proper crop debris cleaning). Biological control agents emerged as an eco-friendly approach to control *M. oryzae* (Chen *et al.* 2019). As an example, *P. fluorescens* was found to produce antifungal antibiotics that can reduce up to 59% leaf blast infection (Gnanamanick and Mew 1992). But working with biocontrol agents requires favorable conditions for their application and ensure viability during rice cultivation.

In summary, *M. oryzae* represent a global threat to food security. Understanding the biology and molecular mechanisms that govern *M. oryzae* infection is crucial to develop new control strategies. As there is a deep public concern about the side effects of chemical fungicides, it is critical to have effective methods to control the rice blast disease in an environmentally friendly way.

In addition to its agricultural relevance, *M. oryzae* emerged as a model organism for studies on plant pathogen interactions. The complete sequence of its genome is available (Dean 2005). Integrated databases with genome, transcriptome and proteome of *M. oryzae* are also publicly available (Genomic Resources of *Magnaporthe oryzae*; GROMO, Thakur *et al.* 2009).The fungus can grow *in vitro* and infection structures can be easily generated. Efficient transformation protocols and mutant collections have been reported (Dean *et al.* 2012; Perez-Nadales *et al.* 2014). Based on its scientific and economic relevance, the rice blast fungus *M. oryzae* was ranked at the top of the list of phytopathogenic fungi (Dean *et al.* 2012).

3. Plant innate Immunity

As sessile organisms, plants have developed several defenses mechanisms to overcome pathogen invasion. Plants have developed basal (constitutive, or passive) and inducible defense responses. Passive strategies consist in generating physical and chemical barriers to block pathogen entry (Nishad *et al.* 2020). Pre-formed physical and chemical barriers constitute the first line of plant defense, including waxy cuticles, rigid cell walls, and antimicrobial secondary metabolites. In addition, plants have evolved a wide variety of inducible defense mechanisms that are triggered upon pathogen recognition (Boller and He 2009; Jones and Dangl 2006). Thus, recognition of pathogen epitopes, known as Pathogen-Associated Molecular Patterns (PAMPs, formerly known as elicitors) by host receptor proteins, or Pattern Recognition Receptors

(PRRs) triggers the activation of a general defense response referred to as PAMP-triggered immunity (PTI) (Bigeard *et al.* 2015; Couto and Zipfel 2016; Li *et al.* 2020). Pathogens have evolved to overcome PTI by secreting virulence factors, also known as effector proteins, into the plant cytoplasm that prevent recognition or suppress host defense (effector-triggered susceptibility, ETS). In turn, microbial effectors (or host proteins modified by these effectors) can be recognized by intracellular receptors encoded by plant *Resistance* (*R*) genes to trigger a rapid and strong host defense called Effector-Triggered Immunity (ETI). ETI is often associated to the hypersensitive response (HR), a specific form of programmed cell death (PCD) at the infection sites to block pathogen invasion. Plant R proteins fall into two classes according to their protein sequence and subcellular localization: i) intracellular receptors, namely cytoplasmic Nucleotide binding-Leucine-Rich repeat domain-containing receptors (NBS-LRRs) and ii) cell surface-localized receptors, namely Receptor-Like Kinases (RLKs) and Receptor-Like Proteins (RLPs) which typically consist on an extracellular ligand-binding domain, a transmembrane domain and an intracellular kinase domain (Monteiro and Nishimura 2018). Thus, an arms race between pathogens and plants occurs by the generation of new effector proteins to suppress ETI in the pathogen side and, in the plant side, the generation of novel plant resistance gene products (R) to overcome pathogens evolution. This model, referred to as the zig-zag model, was originally proposed for interactions with biotrophic pathogens and integrates the functions of PAMPs, PRRs, effectors and R proteins (Jones and Dangl 2006). However, the zig-zag model describes only part of the story. Later on, the invasion model was proposed to replace the zig–zag model in order to accommodate the spectrum of ligands that the plant immune system perceives, including host-derived ligands that are released as a result of cellular damage caused by the invading pathogen (Damage-Associated Molecular Pattens, DAMPs), and the range of plant pathogens interactions (biotrophs, necrotrophs, endophytes and mutualists) (Cook *et al.* 2015). DAMPs usually are cell wall or extracellular protein fragments and peptides (Hou *et al.* 2019a). Several oligosaccharide fragments

derived from plant cell walls activate plant immunity and behave as typical DAMPs (Pontiggia *et al.* 2020).

PTI is a non-specific resistance mechanism that operates against a wide range of pathogens. Typical PTI responses include reinforcement of cell walls and callose deposition, depolarization of the plasma membrane and influx of extracellular Ca^{2+} into the cytosol, generation of reactive oxygen species (ROS), and the activation of phosphorylation signaling cascades in which wall associated kinases (WAKs), mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) participate. These pathogen-induced signaling cascades lead to transcriptional reprogramming of gene expression and induction of defense-related genes, such as *Pathogen-related* (*PR*) genes, some of them exhibiting antimicrobial activity. PTI also includes the production of antimicrobial metabolites (e.g. phytoalexins) (see section 3.2).

The role of plant phytohormones is crucial in plant immunity. Plant hormones are involved in complex networks in order to properly respond to different types of stresses. Hormone signaling networks can act antagonistically or synergistically in a fine-tuned regulation in order to cope to the sensed stress. Salicylic acid (SA), ethylene (ET), jasmonic acid (JA, and JA-derivatives) and abscisic acid (ABA) have been deeply investigated in plant defense responses to pathogen infection (Aerts *et al.* 2021; Zhou and Zhang 2020). Typically, pathogens that need a living host (biotrophs) are more sensible to SA- mediated responses, while pathogens that kill the host cell (necrotrophs) are generally affected by ET-JA mediated responses. Furthermore, SA and SA-derivatives move from local infected tissue to distal tissue inducing the systemic acquired resistance (SAR). After SAR activation, plant may become broadly resistant to different pathogens for an extended period of time. On the other hand, pathogens can highjack the hormone signaling network for their own benefit disrupting plant phytohormone biosynthesis or signaling, diminishing plants immune responses. Thus, several studies have demonstrated that pathogens can generate phytohormone-mimicking

molecules in order to alter host immune responses (Zhang *et al.* 2018b). **Figure 6** illustrates a general model for plant immunity.

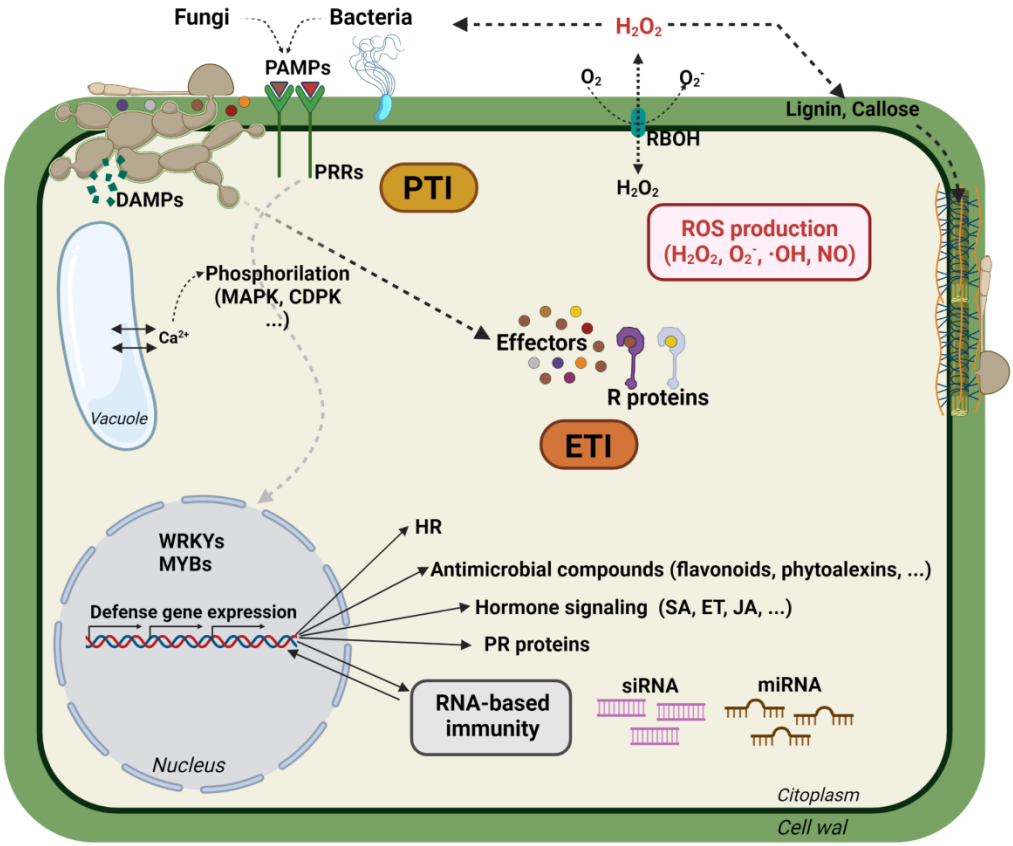

Figure 6. Defense mechanism against fungal and bacterial pathogens in plants. Typical responses to basal (**PTI**) and pathogen-specific (**ETI**) resistance, as well as the RNA-based immune system are represented. **PAMPs**, pathogen associated molecular pattern; **PRR**, pattern recognition receptor; **DAMPs**, damage associated molecular pattern; **MAPKs**, mitogen-activated protein kinases; **CDPKs**, calcium-dependent protein kinases; **RBOH**, respiratory burst oxidase homolog; **ROS**, reactive oxygen species; **HR**, hypersensitive response; **SA**, salicylic acid; **ET**, ethylene; **JA**, jasmonic acid; **siRNA**, small-interfering RNA; **miRNA**, microRNA.

3.1 Reactive oxygen species (ROS) signaling in plant immunity

ROS play an important role in plant immunity. Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) are the three major forms of ROS. In addition, nitric oxide (NO) has been shown to accumulate during HR formation. Among ROS, H_2O_2 is relatively stable and is an important molecule in regulating plant immunity (Torres *et al.* 2006). H_2O_2 might have a direct antimicrobial role against the invading pathogen and also provokes cross-linking of cell wall components to arrest pathogen invasion (Brien *et al.* 2012). ROS also function as signaling molecules for the activation of defense mechanisms, and triggers localized cell death around the infection site. However, when in excess, ROS can be harmful for the plant cell. Thus, ROS can cause oxidation of fatty acids generating toxic lipid peroxides and destabilizing cellular membranes, and damaging nucleic acids and proteins (Sachdev *et al.* 2021). Accordingly, ROS production needs to be tightly controlled in plants. To avoid ROS damage, plants cells use ROS scavenging systems based on enzymatic and non-enzymatic mechanisms. ROS-scavenging enzymes include catalase, superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, peroxiredoxin, thioredoxin, glutaredoxin, glutathione reductase, and dehydroascorbate reductase. Non-enzymatic mechanisms include production of antioxidant molecules like glutathione, flavonoids or carotenoids.

ROS are produced in different cellular compartments during normal metabolism. In the apoplast, ROS production is mediated by plasma-membrane bound NADPH oxidases encoded by *Rboh (Respiratory busrt oxidase homolog)* genes (Torres *et al.* 2002). Plants contain several *NADPH oxidase* genes, 9 in rice which are transcriptionally regulated upon pathogen recognition. The role of iron as a catalytic element in the production of ROS will be discussed in section 4.5.

3.2 Phytoalexins

Phytoalexins are low molecular weight, broad-spectrum antimicrobial compounds involved in plant defense. Phytoalexins are usually synthetized *de novo* upon biotic or abiotic stress so they do not accumulate in uninfected plant tissues (Cho and Lee 2015). Phytoalexin production occurs in response to pathogen infection or elicitor treatment (Duan *et al.* 2014; Hasegawa *et al.* 2010; Okada *et al.* 2007). For many phytoalexins, an antimicrobial activity has been demonstrated, pointing them as key molecules that contribute to limit pathogen invasion (Cho and Lee 2015). From the structural point of view, phytoalexins belong to diverse chemical families including phenolics, terpenoids, furanoacetylenes, steroid glycoalkaloids, sulfur-containing compounds and indole. Amine-conjugated phenolics acids, phenylamides (such as N-trans-cinnamoyltryptamine, N-p-coumaroylserotonin and N-cinnamoyltyramine) typically implicated in cell wall reinforcement have been also shown to exhibit antifungal activity, suggesting that phenylamides might be also considered as phytoalexins.

In rice, the major phytoalexins are diterpenoid phytoalexins and the flavonoid sakuretine (Hasegawa *et al.* 2010). Diterpene phytoalexins are classified into four structurally distinct classes: momilactones (A and B), oryzalexins (A to F), oryzalexin S, and phytocassenes (A to E) (Arruda *et al.* 2016). The diterperne phytoalexin biosynthesis pathway has been elucidated **(Figure 7)**. Diterpenoid phytoalexins are produced via geranylgeranyl diphosphate (GGDP) pathway. The early terpenoid biosynthesis occurs in the plastid via the methylerythritol phosphate (MEP) pathway to form the main diterpenoid phytoalexin precursor, the isopentenyl diphosphate (IPP) and the dimethylallyl diphosphate (DMAPP) (Jeandet *et al.* 2014). Several genes involved in the MEP pathway are induced by elicitor treatment in rice *(OsDXS3, OsDXR, OsCMS, OsCMK, OsMCS, OsHDS* and *OsHDR)* (Okada *et al.* 2007). The expression of these genes has been purposed to be pivotal for the production of high amounts of phytoalexins. GGDP suffers several

cyclization steps to form the hydrocarbons ent-cassa-12,15-diene, ent-sandaracopimaradiene, syn-pimara-7,15-diene, and stemar-13-ene, precursors of the four types of diterpenoid phytoalexins. The genes involved in the hydrocarbons synthesis have been well studied (*OsCPS2*, *OsCPS4*, *OsKSL4*, *OsKSL8*, *OsKSL7* and *OsKSL10)*. Further modifications require a series of several cytochromes P450 (CYPs) to finally produce the four main classes of phytoalexins. The role of phytoalexins in rice defense responses to *M. oryzae* infection has been well studied (Duan *et al.* 2014; Hasegawa *et al.* 2010; Sánchez-Sanuy *et al.* 2019; Umemura *et al.* 2003). Interestingly, the majority of the biosynthetic genes related to diterpene phytoalexin biosynthesis in rice are located in clusters on chromosomes 2 and 4 (operon-like gene clusters) (Miyamoto *et al.* 2016).

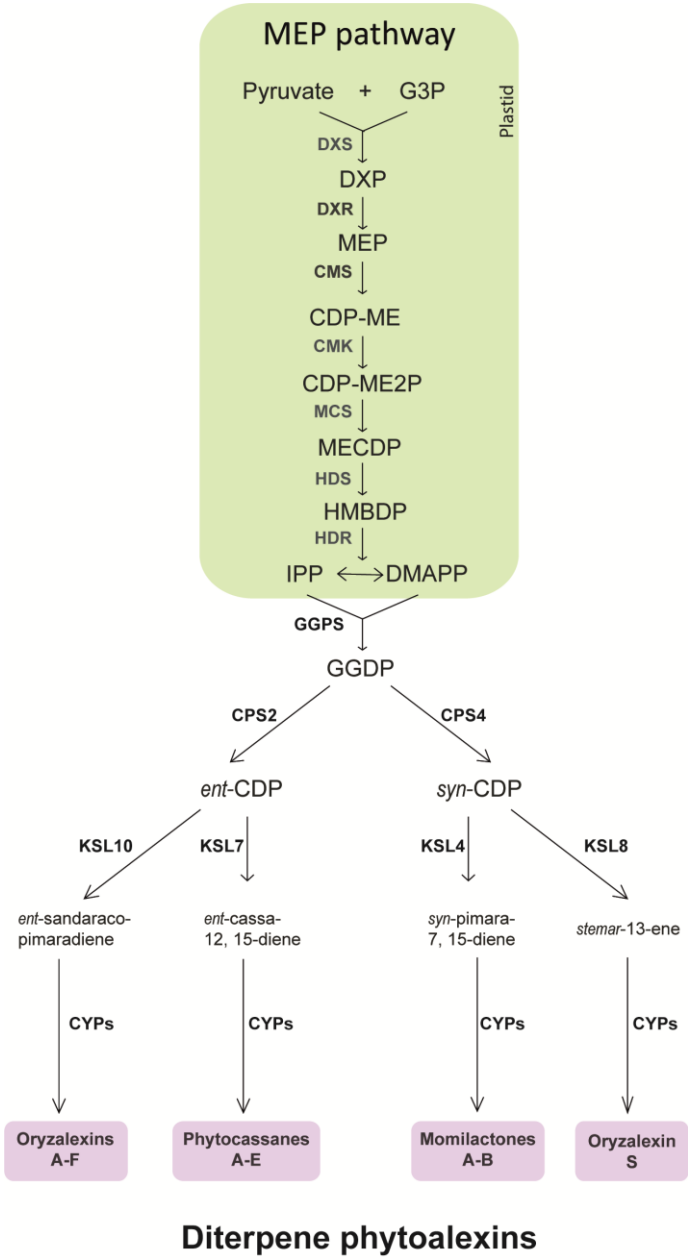

Figure 7. Methylerythritol phosphate pathway (MEP) and diterpenoid phytoalexin pathway. *G3P*, glyceraldehyde-3-phosphate; *DXP*, 1-deoxy-D-xylulose 5-phosphate; *MEP*, 2- C-methyl-D-erythritol 4-phosphate; *CDP-ME*, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; *CDP-ME2P*, 2-phospho-4-(cytidine 5'- diphospho)-2-C-methyl-D-erythritol; *MECDP*, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; *HMBDP*, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; *DMAPP*, dimethylallyl diphosphate; *GGDP*, geranylgeranyl diphosphate; and *CDP*, copalyl diphosphate. Enzymes are indicated in bold.

3.3 Pathogenesis-related proteins

Plant pathogenesis-related proteins (PRs) are a group of diverse molecules usually induced by pathogens. They are key molecules in plant innate immunity and are classical hallmarks of plant defense signaling pathways (Ali *et al.* 2018; Van Loon *et al.* 2006). *PR* genes are highly related to biotic and abiotic stresses and are key components of SAR, aiming to restrict infection and prevent infection to distal parts of the host. For that reason, pathogens have evolved a several effector molecules that target PR proteins during infection (Breen *et al.* 2017). PRs were first described at 1970 in *Nicotiana tabacum* infected by tobacco mosaic virus. First, five major families of PRs were described (PR1, PR2, PR3, PR4, PR5), nowadays there are up to 17 PR families (Ali *et al.* 2018; Van Loon and Van Kammen 1970; Van Loon *et al.* 2006).

PRs are highly resistant to proteases and remain soluble at low pH (Van Loon and Van Kammen 1970). They are widely distributed in plants and accumulate in all plant organs, especially in leaves, upon pathogen infection. PRs can also constitutively accumulate in other parts such as flowers, pollen, stigma, seeds and tubers. Also, senescence is accompanied by the accumulation of specific PR proteins related to leaf aging (Borniego *et al.* 2020). PR proteins are largely distinct among them and are classified based on their biochemical features. They normally accumulate in the plant apoplast or the vacuole. Over-expression of *PR* genes leads to enhanced resistance against phytopathogens (Tang *et al.* 2017; Wu *et al.* 2016). PR proteins are classified in the following families: Antifungal (PR-1 family), β-1,3-glucanases (PR-2 family), Class I, II, IV, V, VI, VII chitinases (PR-3 family), Class I, II Chitinases (PR-4 family), thaumatin-like proteins (PR-5 family), proteinase inhibitors (PR-6 family), endopeptidase (PR-7 family), class III chitinase (PR-8 family), peroxidase (PR-9 family), ribonuclease-like proteins (PR-10 family), class I chitinase (PR-11 family), plant defensins (PR-12 family), thionins (PR-13 family), lipid transfer proteins (PR-14 family), oxalate oxidase (PR-15 family), oxidase-like (PR-16 family) and antifungal and antiviral (PR-17 family) (Ali *et al.* 2018).

3.4 Defense priming

Priming is an adaptive strategy that improves the defensive capacity of plants. The defense priming phenomenon is defined as an induced physiological state marked by an enhanced activation of defense mechanisms (Mauch-Mani *et al.* 2017). Primed plants develop stronger, quicker and/or more sustained defense reactions than non-primed plants resulting in increased resistance and/or stress tolerance (Conrath *et al.* 2015; Martinez-Medina *et al.* 2016). Comparing with constitutive expression of defense responses, defense priming does not consume plant resources and does not have an impact in the host plant energy status, thus, reducing the probability of affecting plant development and yield. Priming can occurs through interindividual or interspecies communication and can be genetically transmitted via epigenetic modifications making priming a transgenerational-inherited mechanism (Jaskiewicz *et al.* 2011; Pastor *et al.* 2013). Several stimuli have been described to induce a primed state such as beneficial microbes, pathogens or arthropods, as well as chemicals and abiotic cues. Colonization of plants by growth-promoting beneficial rhizobacteria (e.g. *Pseudomonas*, *Serratia*, *Bacillus*), fungi (*Trichoderma* spp), nonpathogenic strains of *Fusarium*, and arbuscular mycorrhizal fungi (*Glomeromycota*) can induce systemic resistance (Campos-Soriano *et al.* 2012; Gerlach *et al.* 2015; Jung *et al.* 2012; De Vleesschauwer and Höfte 2009). Herbivore-induced plant volatiles might act as elicitors of priming in distal parts of the plant, as well as in neighboring plants warning of the possible danger (Ton *et al.* 2007).

Priming is being explored as a means of improving disease resistance in plants. Exogenous application of chemical molecules such as β-Aminobutyric acid can induce defense priming (Thevenet *et al.* 2017). Different priming-activating compounds are commercialized (e.g. Primtal "Stress-free technology", https://www.artal.net). Abiotic stimuli can also induce defense priming (Thomas *et al.* 2020). Exposure of *Arabidopsis* plants to a mild heat, cold or salt stress can improve resistance against

virulent *P. syringae* (Singh *et al.* 2014). Other stresses such as wounding, submergence, and exposure to UV light or ozone have been pointed to act as priming triggering factors. The wide range of stresses that plants can suffer, the vast microbial diversity in natural environments and the complexity of priming *per se* makes that the components behind this state of alert remain to be elucidated. Clearly, a better understanding of the molecular and chemical mechanisms underlying priming will allow developing new and promising control strategies.

4. Mineral nutrition in plants

Plant nutrition has been widely studied due to its implications in plant growth and productivity (Pandey 2018). Nutrients can be divided into macronutrients and micronutrients. Macronutrients are those that are required in higher quantities, and include: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulfur (S), and magnesium (Mg). The micronutrients or trace elements include: iron (Fe), boron (B), chlorine (Cl), manganese (Mn), zinc (Zn), copper (Cu), molybdenum (Mo), and nickel (Ni). All nutrients need to be incorporated in a fine-tuned regulated manner and properly distributed across the plant body to maintain an adequate metabolic state. Unbalance of any element can severely affect plant growth and metabolism. For that reason, plants have developed highly specialized mechanisms to maintain nutrient homeostasis.

However, modern agricultural practices have altered natural recycling mechanisms of nutrients, which have led to a scenario of overuse of fertilization practices to maintain crop yields. In addition, several studies have linked the nutritional status of plants with disease resistance (Val-Torregrosa *et al.* 2021). This thesis has focused on how the Fe nutritional status influences rice immune responses and disease. Relevant studies related to other essential nutrients in plants are also discussed.

4.1 Nutrient homeostasis and plant immunity

During plant-pathogen interactions, multiple mechanisms operate in both partners that determine the outcome of the interaction, resistance or susceptibility, including nutrient availability. Hence, nutrient homeostasis must be carefully regulated as host and pathogen compete for nutrients. Mechanisms for maintenance of nutrient homeostasis in the host need to be highly dynamic to allow normal plant growth while providing a way to arrest pathogen growth. This dynamic interaction is particularly relevant when plants are grown under nutrient limiting conditions. On the other hand, an increase in nutrient supply might create a more favorable environment for pathogen growth that would allow the pathogen to improve nutrient acquisition from the host plant, thus, promoting pathogenicity. During an infectious process, plants can modulate nutrient homeostasis by exploding their essentiality or toxicity upon invaders. In animals, withholding strategies are used to restrict iron availability to invading pathogens, a process termed as "nutritional immunity" (Hood and Skaar 2012).

Several works have related the plant nutrient homeostatic state with plant immunity. Both nutrient deficiency and excess might affect disease resistance. As an example, high supply of nitrogen has been associated to increased severity of the rice blast disease, a phenomenon referred to as Nitrogen-Induced Susceptibility (NIS) (Ballini *et al.* 2013). It is also known that N supply has an impact on the two interacting partners: host and pathogen. From the perspective of the host plant, N availability has an effect on plant metabolism which, in turn, might affect host defence responses. From the perspective of the pathogen, NIS to rice blast fungus has been shown to be associated with the induction of rice genes implicated in N recycling and an increase in *M. oryzae* pathogenicity (Huang *et al.* 2017). Contrary to what is observed in rice, N fertilisation reduces disease severity caused by *Verticillium* spp. in *Solanum* species, indicating that no generic model can describe the role of N in a given interaction (Huang *et al.* 2017; Veresoglou *et al.* 2013). Even

though nutrient supply has proven to be an important factor in determining resistance or susceptibility to pathogen infection in the field, the molecular mechanisms by which nutrient stress and immune signaling pathways interact with one another remain poorly understood.

Regarding Pi nutrition, emerging evidence supports the existence of crosstalk between the Pi starvation and immune responses in plants (Castrillo *et al.* 2017; Chan *et al.* 2021). Recently, our group described that high Pi fertilisation compromises the expression of immune responses and enhances susceptibility to infection by *M. oryzae* in rice plants (Campos-Soriano *et al.* 2020). On the other hand, in the last century, copper (Cu) has been widely used as fungicide and bactericide in agriculture (Bordeaux mixture). In addition to have a direct effect on microbial pathogens, Cu has been shown to modulate plant defense responses by activating MAPK signaling leading to upregulation defense genes in rice (Yuan *et al.* 2010). Altogether, these results demonstrated that there is an intimate link between nutrient signaling and immune signaling in plants.

In the frame of this thesis, it was demonstrated that high Fe supply promotes the expression of defense genes and enhances resistance to *M. oryzae* infection (Peris-peris *et al.* 2017).

4.2 Relevance of iron in plant growth and development

Fe is an essential element for plants and essential for many metabolic processes, and a co-factor for a variety of proteins mediating redox reactions. Plants require Fe for photosynthesis, mitochondrial respiration and hormone biosynthesis, DNA synthesis, chlorophyll synthesis, maintenance of chloroplast structure and function, reduction of nitrates and sulfates, among others (Connorton *et al.* 2017). Fe is bound to prosthetic groups necessary for the activity of many enzymes. Cytochromes contain tetrapyrrole rings with Fe as a central atom (heme). Fe can change between oxidation states Fe^{3+} to Fe^{2+} acting as one-

electron carriers fundamental in electron transport chains (Heldt and Piechulla 2021). Fe deficiency results in chlorosis, poor growth and reduced yields. Plants must transport Fe to proper cellular and subcellular locations to prevent the generation of Fe-catalyzed hydroxyl radicals. Along with this, Fe homeostasis must be tightly controlled in plants through very dynamic processes.

Although Fe is a constituent of approximately 5% of Earth's crust (Mengel and Kirkby 2004), the bioavailability of Fe is usually very low, and therefore iron deficiency is one of the biggest problems in agricultural soils worldwide (Colombo *et al.* 2014). Fe deficiency leads to reduced yield and limits nutritional quality of crops. Iron availability depends on soil conditions. In soils with high pH, iron is insoluble (Fe^{3+}), forming hydroxides complexes making it poorly available for living organisms. Under reductive conditions, iron is found under the soluble ferrous form (Fe^{2+}). Fe^{2+} can be harmful as, in the presence of hydrogen peroxide, it can form hydroxyl radicals leading to cell damage. Therefore, iron acquisition, use and storage need to be tightly regulated at the cell and tissue level to provide Fe for plant metabolism while preventing accumulations of deleterious ferrous iron. Either in excess or deficient conditions, iron has hazardous effects in plant growth. In rice, iron toxic effects can reduce yields up to 100% (Becker and Asch 2005). Being rice a staple food for half of the world's population, iron deficiency has a direct impact in rice production, hence, in dietary Fe supply. Iron deficiency anemia is the most common type of anemia (FAO, 2020).

4.3 Regulation of iron homeostasis in plants

Angiosperms have evolved two distinct strategies to overcome low Fe conditions and to take up Fe from the soil, referred to as Strategy I and Strategy II **(Figure 8, left panel)** (Bandyopadhyay and Prasad 2021; Hindt and Guerinot 2012; Kobayashi and Nishizawa 2012). The strategy I, or **reduction strategy**, is used by dicotyledoneous and non-graminaceous monocots species to increase Fe^{3+} solubility (Ishimaru *et al.* 2006; Kobayashi and Nishizawa 2012). The strategy I, involves the secretion of phenolics and soil acidification by the release of protons into the rhizosphere, and is mediated by Phenolics Efflux Zero 1 (PEZ1) and H^{+}-ATPase, respectively (Fox and Guerinot 1998; Ishimaru *et al.* 2011). Iron is subsequently reduced from the ferric (Fe^{3+}) form to a more soluble ferrous form (Fe^{2+}) by a ferric reductase-oxidase (FRO) and then it is transported across the plasma membrane into the root cells by Iron-Regulated Transporter (IRT1, IRT2) (Eide *et al.* 1996; Robinson *et al.* 1997; Vert *et al.* 2001). The Strategy II, also called the **chelation strategy**, is used by graminaceous species. Their roots secrete Fe^{3+} chelators of the mugineic acid (MA) family called phytosiderophores (PS) (Kobayashi and Nishizawa 2012). The MA biosynthetic pathway has been well characterized (Inoue *et al.* 2003; Itai *et al.* 2013). This pathway implies the biosynthesis of the non-proteinaceaous amino-acid nicotianamine (NA) from S-adenosyl-L-methionine by NA synthase (NAS) and then NA is synthesized into 2'-deoxymugineic acid (DMA), the precursor MAs, by NA aminotransferase (NAAT), and DMA synthase (DMAS). Secretion of MAs from rice roots to the rhizosphere is mediated by *OsTOM1* (Nozoye *et al.* 2011). The expression of the genes involved in the Fe^{3+}-MAs transport system is dependent on *OsIRO2* (Ogo *et al.* 2007). The resulting Fe^{3+}-phytosiderophores complexes are translocated into root cells by yellow-stripe like (YSL) transporters in the plasma membrane such as *OsYSL15* transporter (Conte and Walker 2011). Even though rice was originally described as a Strategy II plant, evidence support that rice uses mechanisms from both strategies, Strategy I and Strategy II (Ishimaru *et al.* 2006).

Iron deficiency transcriptionally induces the expression of genes responsible of Fe uptake. This transcriptional re-arrangement is primarily regulated by transcription factors of the ABI3/VP1 family (IDEF1), NAC family (IDEF2) and helix-loop-helix (bHLH) family (Ogo *et al.* 2006; Kobayashi *et al.* 2010a). In addition, Iron-binding Haemerythrin RING ubiquitin ligases (HRZ) and iron man (IMA)/Fe-uptake-inducing peptides are reported to regulate Fe-deficiency responses (Kobayashi *et al.* 2019). Expression of Fe-uptake related genes in roots is co-regulated by two different signals: a shoot-derived long-distance signal and a local signal from the apoplast or the rhizosphere (Kobayashi *et al.* 2019). IDEF1 is pointed to be an upstream regulator of Fe-deficiency responses. IDEF1 also regulate the transcript levels of *OsIMA1* and *OsIMA2*. IDEF1 can bind to Fe^{2+} and together with IDEF2 have the ability to bind to cis-acting iron deficiency–responsive element 1 (IDE1) and IDE2. IDEF1 regulates a large subset of Fe positively regulating Fe-deficiency inducible genes involved in Fe uptake and translocation such as *OsHRZs*, and the bHLH transcription factors *OsIRO2* and *OsIRO3* (Kobayashi *et al.* 2010b). IDEF2 positively regulates another subset of Fe deficiency-inducible genes involved mainly in Fe translocation.

Once Fe enters the cell it must be transported and delivered to the appropriate compartment. **Iron transport** is mainly controlled by chelating molecules and proper control of Fe redox states. Iron is transported into the xylem conjugated with organic acids such as citrate (Kobayashi *et al.* 2019). In the phloem, Fe is often conjugated with NA and MAs (Curie *et al.* 2009; Kaur *et al.* 2021; Yoneyama *et al.* 2015). As xylem cells are dead and phloem cells are alive it is thought that efflux and influx Fe transporters are required in each cell type, respectively (Kobayashi *et al.* 2019). Several YSL transporters are related to Fe-NA long transport (Kobayashi 2019). Rice possesses 18 YSL genes (*OsYSL1–18*) (Koike *et al.* 2004). For instance, *OsYSL2* has been implicated in long transport of NA-chelated Fe (Koike *et al.* 2004). Other two YSL transporters encoded by *OsYSL15* and *OsYSL18* which transport Fe^{3+}-DMA are involved phloem Fe transport (Aoyama *et al.* 2009; Inoue *et al.*

2009). *OsYSL13 and OsYSL16* have been related to Fe homeostasis (Lee *et al.* 2012; Zhang *et al.* 2018a). In Arabidopsis, iron man (IMA) peptides have been suggested to mediate the Fe-deficiency long distance signals transmitted from shoots to roots (Grillet *et al.* 2018).

The chloroplast contains the largest pool of Fe of plant cells, accumulating around the 80 to 90% of cellular Fe (Kobayashi and Nishizawa 2012). This chloroplastic high demand of Fe is required to maintain the proper function of the photosynthetic machinery. The mitochondria also require high amounts of Fe whereas the Mitochondrial Iron Transporter (MIT) has been implicated in mitochondrial Fe transport (Bashir *et al.* 2011).

Other divalent metal transporters may be involved in the iron uptake and translocation across the plant body. Those belong to the ZIP (zinc-regulated transporter, iron-regulated transporter–like protein) and NRAMP (natural resistance-associated macrophage protein). Storage and buffering in cell compartments including the apoplast, organelles and the vacuole allow protection against Fe toxicity (Jeong and Guerinot 2009).

4.4 Fe bioavailability in rice cultivation

Rice is traditionally grown in flooded fields (also known as paddy fields). Fe toxicity occurs in soils with high concentrations of ferrous iron (Fe^{2+}). In paddy fields, microbial activity reduces the insoluble ferric ion (Fe^{3+}) into soluble Fe^{2+} (Becker and Asch 2005). Excessive uptake of Fe^{2+} by the roots causes Fe accumulation at photosynthetic tissues *via* xylem flow. Excess Fe also causes irreversible damages due to the production of radicals that become toxic to the plant cell due to the formation of deleterious ROS via the Fenton reaction (reduction-oxidation between Fe^{3+} and Fe^{2+}) (Stein *et al.* 2014; Wu *et al.* 2014). ROS leads to the oxidation of biomolecules (lipids, proteins, DNA) which might cause multiple damage to cellular structures, and eventually, cell death damage (Becker and Asch 2005). Fe excess symptoms cause red-brownish leaf discoloration also known as "leaf bronzing". Therefore, plants must regulate iron concentration in a homeostatic way to avoid toxicity.

Rice plants have developed different morphological and physiological mechanisms to avoid or tolerate iron toxicity (Becker and Asch 2005). Three main strategies have been purposed to maintain adequate levels of Fe: **(i)** exclusion/avoidance, **(ii)** inclusion/avoidance and **(iii)** inclusion/tolerance **(Figure 8, right panel)**. In the exclusion/avoidance **strategy (i)** Fe^{2+} exclusion occurs in roots which is achieved by oxidation of the iron at the root surface. For this, molecular oxygen is channeled to the roots *via* the aerenchyma to chemically oxidize Fe^{2+} resulting in the formation and accumulation of $Fe(OH)_3$ deposits (known as "iron plaque"). The formation of an iron plaque upon Fe toxicity also generates a physical barrier for further influx of reduced iron. The iron plaque also acts as a "reservoir" of Fe (and other elements) for possible future nutrient deficiencies. Reduced Fe that overcomes the oxidizing barrier can be excluded from root cells by root cell membrane selectivity. In turn, Fe is accumulated in the apoplast of root parenchymatic cells. The inclusion/avoidance **Strategy (ii)** implies Fe incorporation in plant tissues and specific compartmentalization to avoid

Fe damage. Here, Fe is stored in non-photosynthetic tissues to avoid photosynthesis inhibition. Those are called “dumping sites” and include roots, steam and leaf sheath tissues. This overaccumulation in non-photosynthetic tissues may induce the accumulation of phytoferritins. Ferritins are members of the superfamily of iron storage and detoxification proteins present in all living organisms and play important roles in controlling cellular iron homeostasis. Iron-protein complexes and detoxification proteins play important roles in controlling cellular iron homeostasis. In leaves, Fe can be stored in the apoplast to avoid interference with the photosynthetic machinery. Once the excess Fe reaches the symplast the inclusion/tolerance **strategy (iii)** comes in to action. This strategy implies the detoxification of oxidative damage caused by excess Fe and accumulation of phytoferritins to allocate high amounts of Fe. Rice possesses two genes encoding ferritins (*FER1* and *FER2*). Radical scavengers such as gluthatione and cytosolic ascorbate can reduce oxidative stress caused by Fe. Enzymatic activity is also crucial to reduce oxidative stress. Superoxide dismutase (SOD) converts highly reactive superoxide radicals into less harmful H_2O_2. Further detoxification of H_2O_2 is mediated by catalases and peroxidases (discussed in section 1.3).

Rice cultivars differ in their tolerance for iron toxicity (Onyango *et al.* 2019; Pereira *et al.* 2014; Wu *et al.* 2014). Although the capability of rice plants to overcome Fe toxicity is genetically determined, other factors can also be important factors, such as age, climate conditions, or stress intensity and duration. Depending on the mechanisms used by the rice plant to alleviate excess Fe, resistant and sensitive genotypes can be identified. Moreover, rice varieties can be further classified in includer and excluder types depending on leaf iron concentrations (Engel *et al.* 2012). In particular, *O. sativa* var. Nipponbare, a widely used variety in rice research (current reference genome of *japonica* rice), has been classified as a sensitive includer genotype.

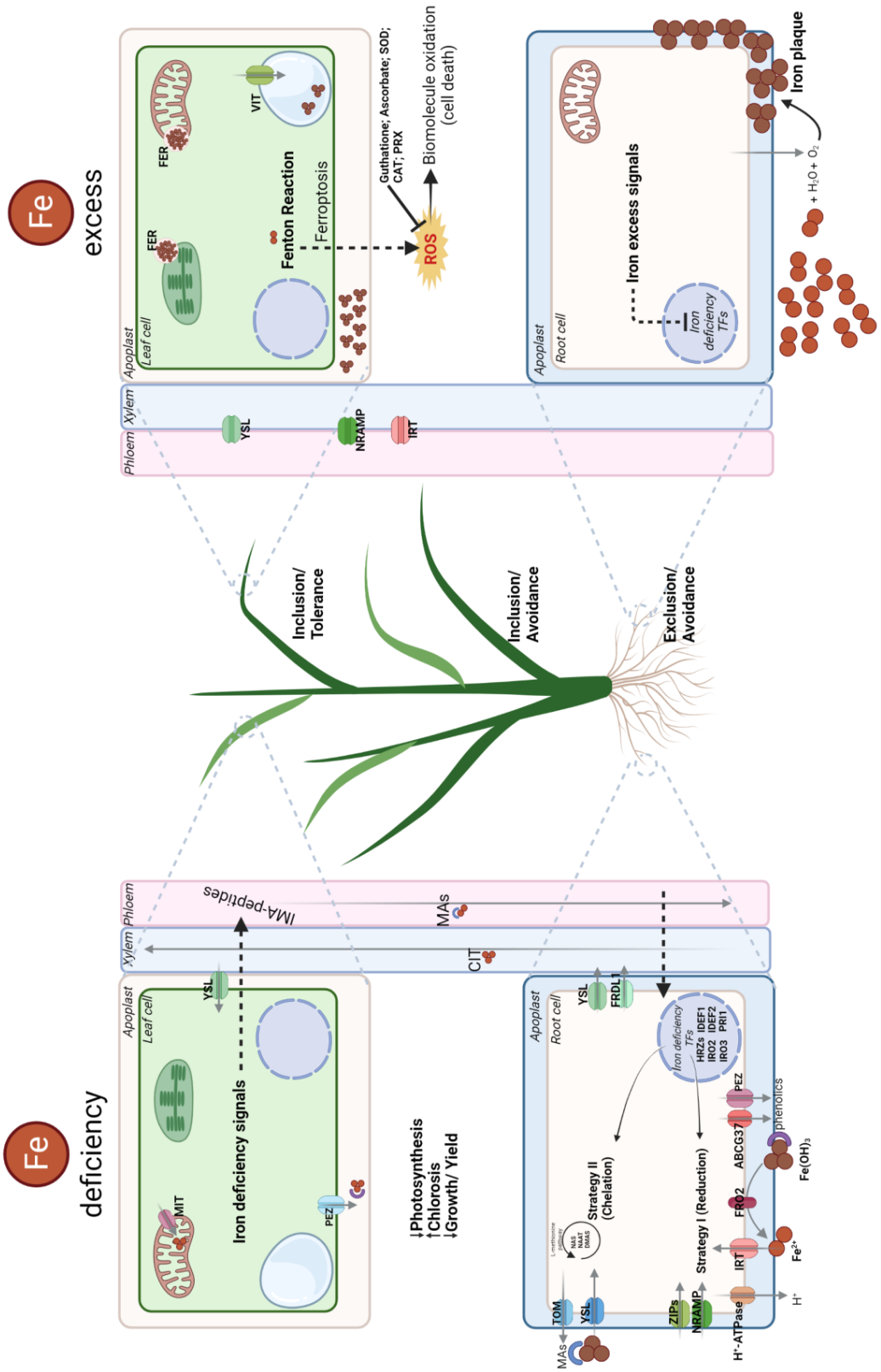
Fe
excess
Apoplast
Leaf cell
FER
FER
VIT
Fenton Reaction
Ferroptosis
Guthatione; Ascorbate; SOD; CAT; PRX
Biomolecule oxidation (cell death)
ROS
Phloem
Xylem
YSL
NRAMP
IRT
Apoplast
Root cell
Iron excess signals
Iron deficiency TFs
Iron plaque
+ H2O + O2
Inclusion/ Tolerance
Inclusion/ Avoidance
Exclusion/ Avoidance
Fe
deficiency
Apoplast
Leaf cell
YSL
Iron deficiency signals
MIT
PEZ
IMA-peptides
MAs
CIT
Xylem
Phloem
↓Photosynthesis
↑Chlorosis
↓Growth/ Yield
Apoplast
Root cell
YSL
FRDL1
Iron deficiency TFs
HRZs IDEF1
IRO2 IDEF2
IRO3 PRI1
PEZ
ABCG37
phenolics
Fe(OH)3
Strategy II (Chelation)
Strategy I (Reduction)
FRO2
IRT
Fe2+
H+
H+-ATPase
ZIPs
NRAMP
TOM
YSL
MAs

Figure 8. Rice plants iron homeostasis deficiency and excess responses. Left panel, iron deficiency responses. Right panel, iron excess responses. **ABCG37**, ABC transporter G family member 37; **CAT**, catalase; **CIT**, citrate; **DMAS**, deoxymugineic acid synthase; **FER**, ferritin; **FRDL**, ferric reductase defective like; **FRO2**, ferric reductase oxidase 2; **HRZ**, Iron-binding haemerythrin RING ubiquitin ligase; **IDEF1** and **2**, iron deficiency-responsive cis-acting element binding factor 1 and 2; **IRO2** and 3, iron-related transcription factor 2 and 3; **IRT**, iron regulated transporter; **IMA**, iron deficiency-inducible peptide- IRON MAN; **MA**, mugineic acid; **MIT**, mitochondrial iron transporter; **NAAT**, nicotianamine aminotransferase; **NAS**, nicotianamine synthase; **NRAMP**, natural resistance-associated macrophage protein; **PEZ**, phenolics efflux transporter; **PRI1**, positive regulator of iron homeostasis 1; **PRX**, peroxidase; **ROS**, reactive oxygen species; **SOD**, super oxide dismutase; **TOM**, transporter of mugineic acid family phytosiderophores; **VIT**, vacuolar mugineic acid transporter; **YSL**, yellow stripe-like; **ZIP**, zinc-regulated transporter.

4.5 Iron homeostasis and plant immunity

Several reports have linked iron homeostasis to plant immunity. For instance, the role of Fe homeostasis in the control of redox-dependent defense responses has been demonstrated in wheat leaves during infection by *B. graminis* (Liu *et al.* 2006). Here, the fungus induces secretion of Fe^{3+} from the host cell to the apoplast, which leads to Fe depletion in the cytosol. In the apoplast, Fe^{3+} accumulates at the cell wall appositions where it mediates the oxidative burst. Hence, H_2O_2 and cytosolic Fe deficiency induces the expression of defense-related genes. Increased resistance is attributed to a major ROS production, coinciding with Fe accumulation at infection sites (Liu *et al.* 2006). Plants might modulate plant defense responses due to its ability to catalyze ROS formation and oxidative stress in plant tissues. A strong oxidative burst is generally associated with resistance (Taheri *et al.* 2014). ROS, in particular H_2O_2, acts as an antimicrobial agent, and also contributes to the strengthening of cell walls. In *Arabidopsis thaliana*, it has been reported that iron deficiency triggers the accumulation of antimicrobial peptides such as scopoletin, a phytoalexin with antifungal activity effective to arrest infection by *Alternaria alternata* (Sun *et al.* 2014). Iron starved *Arabidopsis* plants are more resistant to the necrotrophic pathogens *Dickeya dadantii* and *B. cinerea* (Kieu *et al.* 2012). In other studies, iron-

starved maize plants have been shown to be more susceptible to by *Colletotrichum graminicola* in maize (Ye *et al.* 2014).

In rice, Fe deficiency triggers the secretion of several phenolics including protocatechuic acid which has antimicrobial activity (Tzin and Galili 2010). Interestingly, protocatechuic acid confers resistance to the necrotophic fungal pathogen *C. circinians* in onion (Agrios 2005). Collectively, these pieces of evidence suggest that alterations in iron homeostasis underlie plant defense responses. As both partners, host and pathogen, compete for this micronutrient, control of iron homeostasis is of central importance in host-pathogen interactions. The impact of iron supply on disease resistance appears to be dependent on the host species and the type (e.g. fungus, bacteria) and lifestyle of pathogen (necrotroph, biotroph, hemibiotroph). Results presented in this Ph. D. Thesis support that iron functions as a mediator in the rice response to *M. oryzae* infection.

4.6 Ferroptosis in plant immunity

Ferroptosis is an iron-dependent type of cell death first described in animal cells and more recently in plants (Dangol *et al.* 2019; Distéfano *et al.* 2017; Dixon *et al.* 2012; Kazan and Kalaipandian 2019; Stockwell *et al.* 2017). Ferroptotic cell death differs from apoptosis or necrosis. It is triggered by the iron-dependent accumulation of ROS and accumulation of lipid peroxides (Cao and Dixon 2016). Specifically, highly reactive Fe^{2+} reacts with H_2O_2 to produce Fe^{3+} and ·OH causing lipid peroxidation. Ferroptosis can be promoted by glutathione depletion which is one of the most important antioxidants in cells, and ferroptotic cell death can be inhibited by the application of iron quelators. As the production of apoplastic H_2O_2 is dependent on NADPH oxidases, ferroptotic cell death can be also suppressed by NADPH oxidase inhibitors such as diphenyleneiodonium (DPI). Moreover, NADPH oxidase requires NADP-malic enzyme (NADP-ME) as an electron source

and, accordingly, *nadp-me* mutant rice plants are compromised in the production of H_2O_2 (Dangol *et al.* 2019). The *nadp-me* mutant rice plants are then successfully colonized by *M. oryzae* (Dangol *et al.* 2019). Moreover, actin microfilament reorganization is also required for H_2O_2 and Fe deposition at pathogen penetration sites (Dangol *et al.* 2019).

During the course of this Ph. D. Thesis, Dangol and collaborators (2019) reported that incompatible *M. oryzae*–rice interactions triggered iron and ROS-dependent ferroptotic cell death. Contrary to this, neither Fe nor ROS accumulated in rice during infection with virulent *M. oryzae*, and ferroptotic cell death could not be observed in a compatible rice/*M. oryzae* interaction. These results indicate that iron- and ROS-dependent signaling cascades are involved in ferroptotic cell death in rice and that this process is effective to suppress *M. oryzae* infection. Results presented in this Ph. D. Thesis provide molecular insights into the role of ferroptosis in the rice response to *M. oryzae* infection.

5. Small RNAs in plants

Small RNAs (sRNAs) are short non-coding RNAs that guide gene silencing at the transcriptional and post-transcriptional level in a sequence-specific manner in eukaryotes (Kamthan *et al.* 2015; Singh *et al.* 2018). Two classes of sRNAs are produced in plants: the small-interfering RNAs (siRNAs) and the microRNAs (miRNAs). These two classes of small RNAs are similar in size (20-30 nucleotides in length) but are distinguished by their biogenesis pathway, precursor structures and modes of action (Axtell 2013). Whereas miRNAs derive from single stranded RNA precursors with stem-loop structures, siRNAs arise from double-stranded RNA precursors produced by RNA-dependent RNA polymerases (RDRs). A large number of small RNAs have been identified using high-throughput sequencing of small RNA populations in different plant species and tissues. However, the biological function of most plant small RNAs remain to be determined.

Historically, it was considered that PTI and ETI responses to pathogen infection were based on the transcriptional regulation of protein coding genes and that these mechanisms were independent of RNA silencing (as in antiviral defense). RNA silencing is defined as the suppression of gene expression through nucleotide sequence-specific interactions mediated by RNAs (Baulcombe 2004). At present, it is well known that small RNAs are critical regulators of the plant immune system against bacterial and fungal pathogens (Carbonell 2019; Huang *et al.* 2019; Hudzik *et al.* 2020; Wang and Galili 2019; Zhu *et al.* 2019).

5.1 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a class of non-coding smallRNA (sRNA) that mediate post-transcriptional gene silencing in eukaryotes (Jones-Rhoades *et al.* 2006). MiRNAs play a crucial role in a wide range of developmental processes, including organ polarity and morphogenesis, flowering, shoot and root development, and hormone signaling, among others (Chen 2004, 2009; D'Ario *et al.* 2017; Mallory *et al.* 2004). In rice, distinct miRNAs have been shown to control traits of agronomic importance, such as tiller growth, early flowering, panicle and grain production (Miura *et al.* 2010; Sun *et al.* 2019; Wang *et al.* 2012; Wu 2013; Zhang *et al.* 2013). MiRNAs are also involved in the plant response to biotic and abiotic stress (Baldrich and San Segundo 2016; Li *et al.* 2010; Navarro *et al.* 2006; Staiger *et al.* 2013; Sunkar *et al.* 2012; Weiberg *et al.* 2014). Moreover, the detection of miRNAs in phloem suggests that miRNAs are mobile in plant tissues expanding their range of gene expression regulation (Marín-González and Suárez-López 2012). As an example, long-distance movement of miR399 from shoots to roots has been shown to occur during phosphate starvation responses in *Arabidopsis* (Lin *et al.* 2008).

Plant genomes typically encode hundreds of *MIRNA* (*MIR*) genes, which can be found in intergenic or intragenic regions (e.g. in the intron of annotated genes). Based on their sequences, miRNAs are classified

into distinct families consisting in one or more members which designated by a letter after the miRNA number (e.g. miR812a to miR812w).

MiRNAs act as post-transcriptional regulators of gene expression by triggering cleavage or translational inhibition of target transcripts through near-perfect base pairing between miRNA and target transcripts (Brodersen *et al.* 2008; Llave *et al.* 2002). In the last years, novel regulatory processes have been associated to miRNAs, adding a new layer of complexity to the miRNA regulation network. For instance, some miRNAs have been shown to guide DNA methylation in target genes (Bao *et al.* 2004; Wu *et al.* 2010).

5.1.1 miRNA biogenesis

miRNA are single-stranded RNA molecules of around 20-24 nucleotides (nt) in length. In plants, miRNAs are transcribed *via* DNA-dependent RNA Polymerase II (Pol II) from *MIR* genes as long precursor transcripts (primary miRNAs, or pri-miRNAs) **(Figure 9)**. Pri-miRNA are 5′ capped and 3′ polyadenylated, and possess a stem-loop structure consisting of a terminal loop, an upper stem and lower stem (withholding the future mature miRNA). The pri-miRNAs are processed in two steps by a RNAse III Dicer-like (DCLs), typically DCL1, to produce a pre-miRNA (precursor miRNA) and then a double-stranded duplex, the miRNA-5p/miRNA-3p duplex (previously named miRNA/miRNA*). The HYPONASTIC LEAVES 1 (HYL1) and SERRATE (SE) proteins participate in pre-miRNA processing. DCL-1 produced miRNA duplexes contain 2-nt overhangs at both 3'-ends (Arikit *et al.* 2013; Axtell 2013; Jones-Rhoades *et al.* 2006; Rogers and Chen 2013). Duplexes are methylated at the 3'p end by HUE ENHANCER1 (HEN1) and the functional strand of the duplex is loaded into an ARGONAUTE (AGO)-containing RNA-induced silencing complex (RISC), and transported to the cytoplasm *via* exportin-like protein HASTY (HST). AGO directs miRNAs for cleavage or translational inhibition of target mRNAs

(Bologna *et al.* 2018; Vaucheret 2008). Usually, one of the strands of the miRNA duplex is functional, while the other is degraded but, in some cases, both strands can be functional (Bologna *et al.* 2013; Navarro *et al.* 2006).

Several miRNA clusters have been described in plants, including rice, which can be independently transcribed o simultaneously transcribed into single polycistronic transcripts (Baldrich and San Segundo 2016).

The precursor structures of these polycistronic miRNAs might contain copies of the same miRNA family member (e.g. homologous miRNAs) or unrelated miRNAs (e.g. heterologous miRNAs). Polycistronic miRNAs can generate various miRNAs from a single precursor molecule which then might regulate the expression of different target genes in a coordinated manner (Salvador-Guirao *et al.* 2018; Merchan *et al.* 2009).

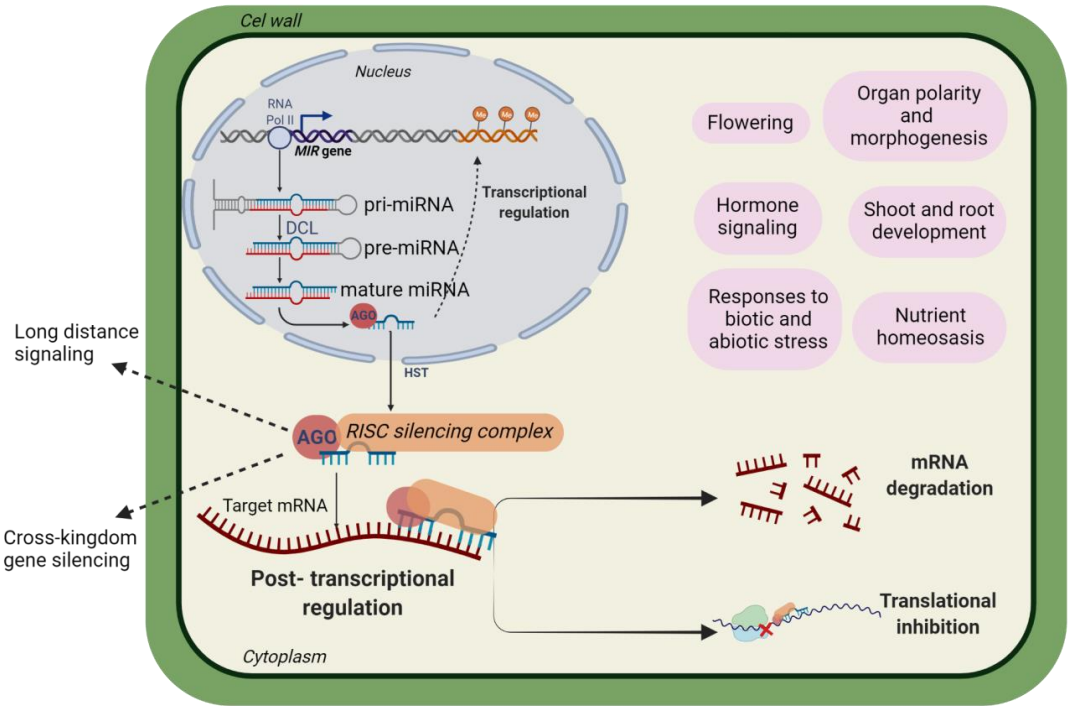

Figure 9. MiRNA biogenesis. Graphical representation of biogenesis, processing and function of miRNAs in plants. **RNA POL II**, RNA polymerase II; **pri-miRNA**, primary miRNA; **pre-miRNA**, precursor miRNA; **DCL**, DICER-LIKE; **HST**, HASTY; **RISC**, RNA-Induced Silencing Complex; **AGO**, ARGONAUTE.

5.1.2 miRNAs: origin and evolution

Plant *MIR* genes are thought to have originated by duplication events that created perfect inverted repeat loci which then evolved by random mutation into short, imperfectly paired, stem-loop structures characteristic of *MIR* genes (Allen *et al.* 2004; Voinnet 2009; Moran *et al.* 2017). The spontaneous evolution from small random inverted repeats in the genome has also been proposed to be the origin of plant miRNAs (De Felippes *et al.* 2008; Nozawa *et al.* 2012; Voinnet 2009). Additionally, transposable elements (TEs) are regarded as an important source of inverted repeats and several miRNAs have been shown to derive from TEs (Cho 2018; Li *et al.* 2011; Piriyapongsa and Jordan 2008; Roberts *et al.* 2014). Whole-genome duplication events, and tandem or segmental duplications of *MIR* genes, contributed to the expansion and diversification of miRNA gene families in plants (Baldrich *et al.* 2018; Maher *et al.* 2006).

RNA-dependent gene silencing is thought to be an ancient process conserved in all eukaryotes and supported by the presence of DLC and AGO proteins in nearly all eukaryotic lineages (Baldrich *et al.* 2018). Some miRNAs are evolutionarily ancient, while other miRNAs evolved recently. Ancient *MIR* genes are preferentially processed by DCL1 to produce canonical 21-nt miRNAs (Kurihara and Watanabe 2004). By contrast, recently evolved miRNAs are processed by DCL3 or DCL4 (rather than by DCL1) to produce miRNAs of variable length, usually 23- to 25-nt long-miRNAs (referred to as long miRNAs or lmiRNA) (Cuperus *et al.* 2011; Nozawa *et al.* 2012; Rajagopalan *et al.* 2006; Vazquez *et al.* 2008). Changes in the fold-back structure of a miRNA precursor have been proposed to determine DCL usage in precursor processing (from DCL3/DCL4 to DCL1) during evolution (Cuperus *et al.* 2011; Nozawa *et al.* 2012)

5.1.3 miRNAs in plant immunity

Typically, PTI and ETI have been considered as protein-based defense mechanisms. However, recent studies demonstrated that miRNAs are key regulators of PTI and ETI (Bundó *et al.* 2020; Wang and Galili 2019; Waheed *et al.* 2021). They can act as positive or negative regulators in plant immunity by targeting negative or positive regulators of defense responses, respectively. Although several studies show that a large population of miRNAs can be regulated by pathogen infection in different plant species, the specific roles of these pathogen-responsive miRNAs remains to be elucidated. The first evidence that miRNAs are implicated in plant immunity came from studies in *Arabidopsis* during infection with the bacterial pathogen *P. syringae* (Navarro *et al.* 2006). Here, perception of the elicitor flg22 induces miR393 accumulation and down regulation of auxin receptors, resulting in resistance to bacterial pathogens (Navarro *et al.* 2006). Since then, other miRNAs controlling diverse processes have been shown to be involved in *Arabidopsis* immunity, either ETI or PTI (Huang *et al.* 2016; Jagadeeswaran *et al.* 2009; Seo *et al.* 2013; Shivaprasad *et al.* 2012; Staiger *et al.* 2013). Some examples are: miR160, miR396, miR398, miR400, miR472, miR773, miR844, miR858, miR863, miR156 (Boccara *et al.* 2014; Camargo-Ramírez, 2017; Lee *et al.* 2015; Li *et al.* 2010; Niu *et al.* 2016; Park *et al.* 2014; Salvador-Guirao *et al.* 2018; Soto-Suárez *et al.* 2017; Yin *et al.* 2019). Most of these studies have been carried out in *Arabidopsis*. Different studies have demonstrated that miRNAs can indirectly regulate the expression of *R* genes (Boccara *et al.* 2014; Li *et al.* 2012; Ouyang *et al.* 2014; Zhai *et al.* 2011). For instance, the miR482 and miR2118 regulate *R* genes of the NBS-LRR type in tomato (Shivaprasad *et al.* 2012).

Previous studies in our group revealed alterations in the accumulation of an important number of miRNAs in rice plants during treatment with elicitors obtained from *M. oryzae* (Baldrich *et al.* 2015; Campo *et al.* 2013). As part of these studies, novel miRNAs from rice have been described (Baldrich *et al.* 2015; Campo *et al.* 2013; Campo *et*

al. 2021; Salvador-Guirao *et al.* 2018). These results, together with those obtained by other groups, support that miRNAs are important components of the rice defense response to *M. oryzae* infection.

At present, only a few miRNAs have been functionally characterized in the rice/*M. oryzae* interaction which can function as positive or negative regulators of rice immune responses. Positive regulators include: miR7695, miR160a, miR162a, miR398b, miR166k-166h and miR812w. Negative regulators are miR156fhl, miR164a, miR167d, miR169a, miR319b, miR396, miR399, miR444b.2, miR439 and miR1873 (Bundó *et al.* 2020; Campo *et al.* 2013; Campo *et al.* 2021; Campos-Soriano *et al.* 2020; Chandran *et al.* 2019; Feng *et al.* 2021; Junhua *et al.* 2021; Li *et al.* 2017, 2019b; Salvador-Guirao *et al.* 2018; Sánchez-Sanuy *et al.* 2019; Zhang *et al.* 2018c; Zhou *et al.* 2020). Relevant for the development of this Ph. D. Thesis was the identification of two novel miRNAs from rice, miR7695 and miR812w (Campo *et al.* 2013; Campo *et al.* 2021). Results obtained in studies related to miR7695 and miR812w are presented in Chapter I and Annex III, respectively.

5.1.4 miRNAs in nutrient homeostasis

Increasing evidence support miRNA regulation of nutrient homeostasis in plants, including rice (Noman and Aqeel 2017; Panda and Sunkar 2015; Paul *et al.* 2015). Some miRNAs are responsive to a specific nutrient, but others can be regulated by different nutrients. In particular, miR167, miR169, miR393, and miR827 have been described as nitrogen-responsive miRNAs, whereas miR395 (targeting ATP sulfurylase genes) functions in sulphur assimilation (Liang *et al.* 2015; Paul *et al.* 2015). Nutrient-responsive miRNAs might then play a role in controlling nutrient homeostasis as well as crosstalk among nutrient stress responses. It is also true that, although a large number of miRNAs have been shown to be responsive to nutrient stress, how such alterations affect nutrient homeostasis remains to be determined.

So far, most research on miRNAs involved in nutrient homeostasis focused on plants under nutrient-limiting conditions, and less is known about their implication in plants that encounter excess of nutrients. Perhaps the best known example is miR399 a key component of the phosphate starvation response (PSR) in plants (Chiou *et al.* 2006). In rice and *Arabidopsis*, miR399 down regulates the expression of *PHOSPHATE2* (*PHO2*) encoding an ubiquitin-conjugating enzyme that mediates degradation of phosphate (Pi) transporters. In this way, miR399 relieves negative post-transcriptional control of Pi transporters for an increase in Pi uptake (Chien *et al.* 2017; Puga *et al.* 2017). Moreover, miR399 moves from roots to shoots and serves as a signaling molecule in regulating Pi homeostasis at the whole-plant level (Pant *et al.* 2008).

5.1.5. miRNA-mediated regulation of iron homeostasis

Some reports indicated alterations in the accumulation of miRNAs in iron homeostatic responses (Carrió-Seguí *et al.* 2019; Kong and Yang 2010; Liang *et al.* 2015). For instance, Arabidopsis miR408 is antagonistically regulated by copper and iron deficiencies, this particular miRNA modulating the expression of laccase-like multicopper oxidase family members *LAC3*, *LAC12*, and *LAC13* (Carrió-Seguí *et al.* 2019). However, the functional evidence for the implication of these miRNAs is still lacking.

Regarding miRNA-mediated regulation of iron homeostasis in rice, studies in our group identified miR7695, a recently evolved miRNA from rice involved in resistance to infection by the blast fungus *M. oryzae* (Campo *et al.* 2013). MiR7695 targets an alternatively spliced transcript of the *Nramp6* (*Natural resistance-associated macrophage pathogen 6*) gene in rice. Most importantly, miR7695 overexpression confers resistance to *M. oryzae* infection (Campo *et al.* 2013).

The Natural Resistance Associated Macrophage Protein (NRAMP) proteins represent a family of plant metal transporters that are

present in a wide range of organisms, from bacteria to humans (Cellier *et al.* 1995). The different plant NRAMP transporters differ in their selectivity and are capable of transporting several metal ions such as iron, manganese, cadmium or zinc. The best characterized plant NRAMP proteins, the *Arabidopsis* AtNRAMP3 and AtNRAMP4 proteins, are required to mobilize vacuole iron stored during germination (Thomine *et al.* 2003). AtNRAMP3 and AtNRAMP4 are also involved in sensitivity to heavy metals such as cadmium. A relationship between the expression of *Nramp* genes and pathogen infection was described in *Arabidopsis*. Here, AtNRAMP3 and, to a lesser extent, AtNRAMP4 are involved in resistance against the bacterial pathogen *Erwinia chrysanthemi* (Segond *et al.* 2009). Chapter I and Annex I and II include results obtained in studies related to miR7695 and *Nramp6* as regulators of iron homeostasis and rice immunity.

5.2. Small interfering RNAs (siRNAs)

Unlike miRNAs that are generated from single-stranded RNA precursors that adopt stem-loop structures, siRNAs derive from double-stranded RNA precursors (dsRNAs) produced by RNA-dependent RNA polymerases (RDRs). The class of siRNAs (sometimes known as short interfering RNAs) includes different types of small RNAs, such as heterochromatic siRNAs (hc-siRNAs), phased secondary siRNAs (pha-siRNAs), trans-acting siRNAs (ta-siRNAs), and natural antisense siRNAs (nat-siRNAs) (Borges and Martienssen 2015; Deng *et al.* 2018). Of them, hc-siRNAs (24-nt in length) are the most abundant siRNAs in plants and participate in transcriptional gene silencing through RNA-directed DNA methylation. The dsRNA precursors are processed by DCL proteins giving rise to distinct types of siRNAs. The production of each type of siRNAs from the corresponding precursor requires the participation of specific members of the RDR family of protein (RDR2, RDR6), and DCL proteins (DCL2, DCL3, or DCL4). For example, hc-siRNAs require RDR2 and DCL3, while production of ta-siRNAs require RDR6 and

DCL4. RDR6, DCL1, and DCL2 are involved in the production of nat-siRNAs. The siRNAs are also loaded into AGO-containing RISC to perform their function. A specificity is also observed in the role of members of the AGO family and the different types of small RNAs. While AGO1 preferentially recognizes miRNAs, AGO4 recognizes hc-siRNAs.

5.3 Small RNAs in cross-kingdom communication

Small RNAs can move between organisms triggering cross-kingdom sequence-specific suppression of gene expression known as RNA interference (Transkingdom RNAi) (Knip *et al.* 2014). This phenomenon has been observed in both plants and animals interacting with pathogenic and symbiotic organisms (Hou *et al.* 2019b; Wang *et al.* 2017b; Weiberg *et al.* 2013, 2014). Small RNAs can traffic from humans to the malaria causal agent *Plasmodium falciparum*, from bacteria to nematodes, from plants to microbes, plants to nematodes, plants to insects, and from fungal pathogens to plants (Zeng *et al.* 2019). In plant/pathogen interactions, there is increasing evidence that both plants and fungal pathogens use cross-kingdom RNAi strategies for their own benefit **(Table 2)** (Wang and Dean 2020).

Naturally occurring cross-kingdom RNAi between plants and pathogens was observed for the first time in *Arabidopsis* and tomato plants infected with the necrotrophic fungus *B. cinerea*. Here, fungus-derived small RNAs enter into the plant cell and hijack the plant RNA interference machinery for suppression of immune responses (Weiberg *et al.* 2014). Here, siRNAs from *B. cinerea* derived from transposon loci, intergenic regions or protein coding loci hijack the host RNAi machinery by binding to Argonaute 1 (AGO1) (Weiberg *et al.* 2013). *B. cinerea* also secretes a small RNA (Bc-siR37) that is predicted to target several *Arabidopsis* genes, including WRKY transcription factors, receptor-like kinases, and cell wall-modifying enzymes (Wang *et al.* 2017b).

In other studies, *Phytophthora* infection was accompanied by an increase in diverse secondary siRNAs in *Arabidopsis* (Hou *et al.* 2019b). Instead of regulating endogenous plant genes, these siRNAs were found to target genes in *Phytophthora* during natural infection. Interestingly, *Phytophthora* evolved to overcome the host defense by producing effectors that specifically inhibiting secondary siRNA biogenesis (suppressors of RNAi) (Hou *et al.* 2019b). Moreover, *Arabidopsis* mutants defective in secondary siRNA biogenesis were hypersusceptible to *Phytophthora* infection. These findings highlight a defense/counter-defense arms race centered on trans-kingdom gene silencing between hosts and pathogens.

These observations support that pathogen-derived siRNAs can translocate into host cells to silence host immunity, thus, acting as effector molecules. Movement of small RNAs has been described in different plant/pathogen interactions **(Table 2)**.

Although most studies so far carried out focused on cross-kingdom trafficking of siRNAs, evidence also supports movement of miRNAs (or miRNA-like species) from a fungus to its host and *vice versa*. For instance, *Puccinia striiformis* f. sp. *tritici* (*Pst*) generates a microRNA-like RNA (milRNA) capable of suppressing wheat defenses by inhibiting the expression of a β-1,3-glucanase (SM638, a *PR2* family member) for suppression of host immunity (Wang *et al.* 2017a).

MiRNAs movement was also described in plant-parasitic plant interactions. Specifically, miRNAs from the parasitic plant *Cuscuta campestris* move to *A. thaliana* during parasitism, resulting in cleavage of host transcripts and secondary siRNA production (Shahid *et al.* 2018). These miRNAs might well function as trans-species regulators of host gene expression.

Interestingly, host plants can also respond to pathogen attack by exporting specific miRNAs to induce cross-kingdom gene silencing in the pathogen, thereby resulting in disease resistance (Yang *et al.* 2021). In

particular, miR166 and miR159 were found to accumulate in cotton (*Gossypium hirsutum*) plants infected with the fungus *Verticillium dahlia* which are then transported to the fungal hyphae to reduce the expression of genes essential for fungal virulence (*isotrichodermin C-15 hydroxylase*, and *Clp-1* encoding a Ca^{2+}-dependent cysteine protease) (Zhang *et al.* 2016).

Collectively, these results support the significant contribution of small RNAs and RNAi to the communication between hosts and pathogens.

	Delivering organism	Target organism	miRNA	Target gene	Reference	
Naturally occurring	*Arabidopsis thaliana*	*Botrytis cinerea*	*miR166*		Cai et al., 2018	From plants to pathogens
			TAS1c-siR483	*Vacuolar protein sorting 51 (BcVps51);Dynactin complex (BcDCTN1)*		
			TAS2-siR453	*Supressor of actin (SAC1)*		
			IGN-siR1			
		Phytophtora infestans	*siRNA-1310*	*Phyca_554980*	Hou et al., 2019	
	Triticum aestivum	*Fusarium graminearum*	*miR1023*	*Alpha/ beta hydrolase (FGSG_03101)*	Jiao and Peng, 2018	
	Gossypium hirsutum	*Verticillum dahliae*	*miR166*	*Ca^{2+} dependent cysteine protease (Clp-1)*	Zhang et al., 2016	
			miR159	*Isotrichodermin C-15 hydroxylase (HiC-15)*		
Transgenic plants	*Nicotiana tabacum*	*Myzus persicae*	*amiRNA*	*Acetylcholinesterase 2 (MpAChE2)*	Guo et al., 2014	
		Helicoverpa armigera	*amiR-24*	*Chitinase*	Agrawal et al., 2015	
	Arabidopsis thaliana	*Helicoverpa armigera*	*HaAce1-amiR1*	*Acetylcholinesterase (HaAce1)*	Saini et al., 2018	
	Oryza sativa	*Chilo suppressalis*	*csu-novel-miR15*	*CsSpo CsEcR*	Jiang et al., 2017	
	Solanum tuberosum	*Phytophthora infestans*	*amiRNA*	RXLR effector *Avr3a*	Thakur et al., 2015	
Naturally occurring	*Botrytis cinerea*	*Arabidopsis thaliana*	*Bc-siR3.1*	*peroxiredoxin (PRXIIF)*	Weiberg et al., 2014	From pathogens to plants
			Bc-siR3.2	*Mitogen activated protein kinase (MPK1and MPK2)*		
			Bc-siR5	*Cell wall-associated kinase (WAK)*		
		Solanum lycopersicum	*Bc-siR3.2*	*MAPKKK4*		
		Arabidopsis thaliana	*Bc-siR37*	Several genes	Wang et al., 2017b	
	Puccinia striiformis f. sp.*tritici*	*Triticum aestivum*	*Pst-milR1*	*β-1,3-glucanase (PR2)*	Wang et al., 2017a	

Table 2. Movement of small RNAs between plants and fungi

5.3.1. Host-induced gene silencing (HIGS)

The trafficking of small RNAs is bidirectional: pathogens can deliver small RNAs to the host plants and plants can deliver small RNAs into invading fungal pathogens. In this way, cross-kingdom RNAi allows the plant to silence target genes in the pathogen via RNAi. This phenomenon has been exploited for the development of host-induced gene silencing (HIGS), a RNA-based technology that has been

successfully used for the control of fungal diseases (Guo *et al.* 2014; Koch *et al.* 2013; Nowara *et al.* 2010; Panwar *et al.* 2018; Song and Thomma 2018; Wang and Dean 2019). Conceptually, HIGS involves plant transgenes that produce siRNAs which can silence a targeted pathogen. As an example, reduced disease symptoms were observed in transgenic *Arabidopsis* and tomato plants expressing siRNA-generating double-stranded RNAs (dsRNAs) targeting *B. cinerea* genes (Wang *et al.* 2016). While delivery of dsRNA/siRNAs by transgenic expression and HIGS have proven to be effective for disease control, it requires the generation of transgenic crop plants which may cause substantial delay for application strategies depending on the transformability and genetic stability of the crop plant species. In addition to being technically challenging in certain crop species, there is still a concern for consumers on transgenic plants in agricultural productions.

Regarding the mechanisms underlying movement of small RNAs between plants and fungi, studies in different plant hosts and their interacting organisms indicated that extracellular vesicles (or exosomes) can be vehicles of small RNA exchange in cross-kingdom RNAi. Extracellular vesicles are important for plant immunity as they contain antimicrobial compounds, such as phytoalexins or phenolics that are routinely produced in plants as a response to pathogen infection (Kwon *et al.* 2008). Furthermore, transportation of small RNAs through exosomes might ensure stability during RNA transfer. Supporting this notion, Baldrich and colleagues reported that plant extracellular vesicles contain diverse small RNA species (Baldrich *et al.* 2019). HIGS might well be based on the uptake of exosome-like vesicles, containing transgene-derived siRNAs from the host (Cai *et al.* 2018). How specific small RNAs are sorted for secretion and absorption remain unknown.

5.3.2. Spray-induced gene silencing (SIGS)

Several studies have demonstrated that the exogenous application of dsRNAs or siRNAs can regulate gene expression in certain pathogens (Dalakouras *et al.* 2019; Koch *et al.* 2016; Wang *et al.* 2016). Wang and collaborators (2016) described a reduction in disease symptoms caused by *B. cinerea* infection by externally applying dsRNAs and siRNAs to fruits (tomato, strawberry, grape), flower petals (rose) and vegetables (onion epidermis, lettuce) (Wang *et al.* 2016). In other studies, spray application of a long non coding dsRNA targeting *F. graminearum cytochrome P450* genes reduced disease development in *F. graminearum*-inoculated barley leaves (Koch *et al.* 2016). Here, it should be noted that studies so far carried out have been focused on the application of dsRNAs or siRNAs derived from them.

The mechanisms of RNA delivery into fungal cells in SIGS are not fully understood. Exogenous RNAs can either be directly internalized into fungal cells or indirectly *via* passage through plant tissue before transport into the pathogen. In the agronomically important barley/*F. graminearum* pathosystem, it was demonstrated that spray application of a long noncoding dsRNA (791 nt *CYP3*-dsRNA), which targets the three fungal cytochrome P450 *lanosterol C-14α-demethylases* that are required for biosynthesis of fungal ergosterol, inhibits fungal growth in the directly sprayed (local) as well as in distal, non-sprayed parts of detached leaves (Koch *et al.* 2016). This observation suggests that sprayed dsRNAs taken by plant cells can move systemically through the vascular system. Clearly, the effectiveness of SIGS relies on extracellular RNA stability and RNA uptake efficiency by pathogens. The use of artificial vesicles or liposomes might help in protecting RNAs in SIGS strategies. This is an expanding area of research.

6. Perspectives and biotechnological applications of miRNAs

MiRNAs have been shown to modulate plant immune responses. The activation or repression of *MIR* genes might then serve to develop traits of interest in crop species, including disease resistance. Studying the biological function of miRNAs will provide valuable information on mechanisms of posttranscriptional gene regulation of immune responses with applications in crop protection. Studies in different plant/pathogen interactions indicate that the function of disease-related miRNAs is very much dependent on the target gene for a particular miRNA, as well as on the host and pathogen species. As previously mentioned, disease resistant transgenic rice have been produced by overexpressing miR7695 (Campo *et al.* 2013). Opposite to this, overexpression of miR399 increases susceptibility to the rice blast fungus (Campos-Soriano *et al.* 2020). These observations support the potential of *MIR* genes, and their corresponding target genes, for disease resistance in rice. Also, depending on the regulatory role of the miRNA/target gene pair, strategies to enhance or suppress the expression of either the *MIR* gene or its target gene should be taken into account (e.g. miRNAs can be used to silence genes that negatively regulate plant resistance). As alterations on the expression of a miRNA might lead to undesirable pleiotropic effects (e.g. fitness penalty), a better knowledge of the mechanism governing miRNA function is required to avoid potential undesirable trade-off effects in transgenic plants with altered expression of miRNAs.

From a technical point of view, the use of miRNA-based technologies provides a smart solution for sequence-specific cleavage of any designated target transcript with a great potential for biotechnological approaches for crop protection. Artificial miRNA (amiRNA) uses miRNA precursors where the miRNA-5p/miRNA-3p sequences have been replaced by an artificial amiRNA-5p/amiRNA-3p sequence that guides silencing of a target gene of interest (McHale *et al.* 2013; Tiwari *et al.* 2014). Artificial miRNAs have been reported to function in silencing

reporter genes, endogenous genes, non-protein coding RNA, and viruses either at the tissue level or at the whole plant (Alvarez *et al.* 2006; Eamens *et al.* 2011; Niu *et al.* 2006; Qu *et al.* 2007; Schwab *et al.* 2006).

The target mimicry is an endogenous regulatory mechanism that plants use to negatively regulate the activity of specific miRNAs. Here, the plant produces a long non-coding RNA containing a motif with sequence complementarity to a given miRNA in which pairing is interrupted by a mismatched loop at the expected miRNA cleavage site that abolishes the cleavage effect. The best known example is *IPS1* (*Induced by Phosphate Starvation1*) which contains a sequence complementary to miR399 but with a mismatched loop at the miR399 recognition site. *IPS1* transcripts sequester miR399, thus, preventing cleavage of transcripts for its target gene *PHOSPHATE 2* (*PHO2*) (Franco-Zorrilla *et al.* 2007). The fact that a non-cleavable miRNA target sequence is capable of inhibiting the activity of a complementary miRNA had major implications for the functional analysis of plant miRNAs through the production of transgenic plants expressing artificial target transcripts that cannot be cleaved by a miRNA of interest. The artificial target mimicry strategy has proven to be an useful tool to unravel the function of several miRNAs in *Arabidopsis* immunity, such as miR396, miR773 and miR858 (Camargo-Ramírez *et al.* 2017; Salvador-Guirao *et al.* 2018; Soto-Suárez *et al.* 2017). As a matter of interest, targeted genome editing technologies represent powerful tools to delineate the function of plant miRNAs. CRISPR has proven to be effective in miRNA editing (Bi *et al.* 2020; Campo *et al.* 2021; Chung *et al.* 2020; Lian *et al.* 2021; Miao *et al.* 2013; Zhou *et al.* 2017).

Small RNA movement and cross-kingdom RNAi between plant and pathogens, is an exciting area of research with applications in crop protection. The mobility of small RNA molecules from the perspective of trans-kingdom gene silencing through RNAi not only has important implications in the biology of plant/pathogen interactions, but also has the potential to become an important method for disease control in the future.

In particular, HIGS designed to target fungal genes that are essential for pathogenesis has emerged as a promising strategy to improve plant resistance to infection by different pathogens, in both model and crop species (Cheng *et al.* 2015; Ghag *et al.* 2014; McHale *et al.* 2013; Pliego *et al.* 2013; Song and Thomma 2018). Despite this, HIGS implies genetic modification of plant hosts. Nowadays, there is a social concern about genetic modified organisms (GMO) and for that reason, several countries or communities, as the European Union, have banned its cultivation.

Spray-induced gene silencing (SIGS) has generated a new concept of environmentally friendly fungicides and opens a new window of novel biotechnological approaches to silence virulence or pathogenicity genes in fungal pathogens avoiding the generation of GMO. Future development and application of new generation of RNA-based fungicides will be an important research direction to control plant diseases. In this Ph. D. Thesis, I provide new insights into the potential of miRNAs as antifungal agents for the control of the blast rice disease.

Last, but not least, as current knowledge and techniques evolve, we anticipate that miRNAs will serve as useful tools in breeding programs as biomarkers for disease resistance. However, the research of miRNAs as biomarkers is still in its early stages, as it requires a better knowledge of miRNA function in plant immunity.

Objectives

The general aim of the Ph. D. Thesis was to investigate the contribution of miR7695 and its target gene, the *Nramp6* gene, in the regulation of iron homeostasis and immune responses in rice, and to explore the feasibility of using miRNAs as a strategy for protection of rice plants against the blast fungus. The specific objectives of this work were:

1. To functionally characterize miR7695 in the regulation of iron homeostasis and immune responses in rice plants. Transcriptomic analysis of mock-inoculated and *M.oryzae*-inoculated wild-type and *MIR7695* activation plants (*MIR7695*-Ac plants) was used to elucidate the role or miR7695. Results are presented in Chapter I.

2. To investigate transcriptional alterations in the response of rice plants to iron treatment and *M. oryzae* infection. To better understand the role of iron in blast resistance I performed transcriptomic analysis in rice plants treated with different iron supply and infected with *M. oryzae*. Results are presented in Chapter II.

3. To explore whether miRNAs can be used as antifungal agents based on its potential for silencing fungal genes upon topical application on rice leaves. Two genes involved in *M. oryzae* development and pathogenicity were selected as the target genes. Complementary approaches were used to produce miRNA molecules (chemical synthesis of miRNA duplexes, *in vivo* synthesis in the heterologous system of *Nicotiana benthamiana*, and *in vitro* synthesis of miRNA precursors). Results are presented in Chapter III.

Chapter I

Osa-miR7695 enhances transcriptional priming in defense responses against the rice blast fungus

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ABSTRACT

Background: MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level in eukaryotes. In rice, *MIR7695* expression is regulated by infection with the rice blast fungus *Magnaporthe oryzae* with subsequent down-regulation of an alternatively spliced transcript of *natural resistance-associated macrophage protein 6* (*OsNramp6*). NRAMP6 functions as an iron transporter in rice.

Results: Rice plants grown under high iron supply showed blast resistance, which supports that iron is a factor in controlling blast resistance. During pathogen infection, iron accumulated in the vicinity of *M. oryzae* appressoria, the sites of pathogen entry, and in cells surrounding infected regions of the rice leaf. Activation-tagged *MIR7695* rice plants (*MIR7695*-Ac) exhibited enhanced iron accumulation and resistance to *M. oryzae* infection. RNA-seq analysis revealed that blast resistance in *MIR7695*-Ac plants was associated with strong induction of defense-related genes, including pathogenesis-related and diterpenoid biosynthetic genes. Levels of phytoalexins during pathogen infection were higher in *MIR7695*-Ac than wild-type plants. Early phytoalexin biosynthetic genes, *OsCPS2* and *OsCPS4*, were also highly upregulated in wild-type rice plants grown under high iron supply.

Conclusions: Our data support a positive role of miR7695 in regulating rice immunity that further underpin links between defense and iron signaling in rice. These findings provides a basis to better understand regulatory mechanisms involved in rice immunity in which miR7695 participates which has a great potential for the development of strategies to improve blast resistance in rice.

INTRODUCTION

Plants have a sophisticated innate immune system for protection against pathogen infection (Doughari 2015; Jones and Dangl 2006). The activation of plant defense responses against pathogens occurs via the recognition of conserved pathogen-associated molecular patterns (PAMPs; previously known as elicitors) by host pattern-recognition receptors (PRR) which, in turn, triggers a signaling cascade leading to the activation of defense-related responses. Pathogen-induced defense responses include the production of reactive oxygen species (ROS), the activation of protein phosphorylation/dephosphorylation cascades, and the production of pathogenesis-related (PR) proteins, among others (Boller and Felix 2009; Macho and Zipfel 2014). Successful pathogens, however, have developed countermeasures to suppress this basal defense in certain plant species and promote disease by delivering effectors into the host. Plants have also evolved Resistance (R) genes that recognize microbial effectors to activate a much stronger immune response, the so called effector-triggered immunity (Cui *et al.* 2015). PTI and ETI have long been considered protein-based mechanisms. However, increasing evidence supports that microRNAs (miRNAs) are also important players in both PTI and ETI (Boccara *et al.* 2014; Li *et al.* 2012; Li *et al.* 2010; Liu *et al.* 2014; Navarro *et al.* 2006; Shivaprasad *et al.* 2012).

MiRNAs are small noncoding RNAs that modulate gene expression in eukaryotes by triggering sequence-specific cleavage or translational repression of target genes (Jones-Rhoades *et al.* 2006). Plant miRNAs play a crucial role in the control of developmental processes and adaptation to environmental stresses, both abiotic and biotic stresses (Baldrich and San Segundo 2016; Chen 2004; Li and Zhang 2016; de Lima *et al.* 2012). Although numerous miRNAs have been reported to be regulated during pathogen infection, the biological role of most of them remains unknown. Furthermore, these studies have been conducted mainly in the model dicotyledonous plant *Arabidopsis thaliana* during interaction with the bacterial pathogen *P. syringae*. Further experimental

validation is required to better understand the regulatory roles of miRNAs in plant immunity.

In the past few years, studies have demonstrated that miRNAs act as regulators of nutrient homeostasis in plants by modulating the expression of genes involved in nutrient homeostasis (Paul *et al.* 2015). It has been shown that miR399 and miR395 play a fundamental role in phosphate and sulfur homeostasis in plants (Chiou *et al.* 2006; Liang *et al.* 2010). Plant miRNAs controlling nutrient homeostasis may also be important factors in controlling disease resistance. Unfortunately, miRNA-mediated mechanisms involved in disease resistance and nutrient homeostasis have been studied separately.

Iron (Fe) is an essential microelement for plant growth required for essential redox reactions in metabolism. Fe is also required for photosynthesis and maintenance of chloroplast function (Abadía 1992). However, excess Fe generates reactive oxygen species (ROS), which might cause oxidative damage to macromolecules (e.g. nucleic acids, lipids, proteins) and cellular structures (Dangol *et al.* 2019; Huang *et al.* 2019; Marcec *et al.* 2019; Naranjo-Arcos and Bauer 2016; Torres 2010). During pathogen infection, Fe homeostasis must be carefully regulated as the host and pathogen compete for the available Fe. The pathogen must acquire this vital element from host tissues, whereas the host plant can deprive the invader of Fe as a defensive strategy. Mechanisms for maintaining Fe homeostasis need to be highly dynamic in the host plant to allow normal plant growth. Although distinct miRNAs have been shown to be responsive to Fe stress (Agarwal *et al.* 2015; Kong and Yang 2010; Paul *et al.* 2016; Valdés-López *et al.* 2010; Waters *et al.* 2012), how such alterations will affect Fe homeostasis and disease resistance remain to be determined.

Rice is one of the most important cereal crops in the world and the model plant for genomics research of monocotyledonous (Goff *et al.* 2002; Yu *et al.* 2002). Rice production is severely affected by blast disease caused by the fungal pathogen *Magnaporthe oryzae* (Wilson and

Talbot 2009). miRNAs controlling traits of agronomic importance (e.g., tiller growth, early flowering, grain production) (Miura *et al.* 2010; Wang *et al.* 2012; Zhang *et al.* 2013) and tolerance to abiotic stress (drought, salinity and cold stress) (Fang *et al.* 2014; Wang *et al.* 2014; Yang *et al.* 2017) have been described in rice. Evidence also supports variations in the accumulation of rice miRNAs during *M. oryzae* infection or treatment with *M. oryzae* elicitors (Baldrich *et al.* 2015; Campo *et al.* 2013; Li *et al.* 2014; Li *et al.* 2016), but the biological function of only a few of these pathogen-regulated miRNAs has been demonstrated. They include both positive regulators (miR7695, miR160, miR398, and polycistronic miR166k-166h) and negative regulators (miR164a, miR169 and miR319) of defense responses against the rice blast fungus *M. oryzae* (Campo *et al.* 2013; Li *et al.* 2014, 2017; Salvador-Guirao et al 2018; Wang *et al.* 2018; Zhang *et al.* 2018c). Hence, to obtain a comprehensive understanding of the regulatory functions of miRNAs in the rice response to *M. oryzae* infection, intense experimental validation of miRNA functioning is mandatory.

We previously reported that the rice miR*7695* is involved in blast resistance (Campo *et al.* 2013). This particular miRNA targets an alternatively spliced transcript of *OsNramp6* (*natural resistance-associated macrophage pathogen 6*), in particular the shortest transcript variant (*OsNramp6.8),* encoding an Fe and manganese transporter, the NRAMP6 protein (Peris-peris *et al.* 2017). Here we show that Fe accumulates at the sites of pathogen penetration (appressoria) and cells surrounding the infection sites in *M.oryzae*-infected rice leaves. *MIR7695* activation results in blast resistance which was associated with local iron accumulation at the infection sites and superinduction of *PR* and diterpenoid phytoalexin biosynthesis genes. Consequently, *MIR7695* activation plants accumulated major rice phytoalexins in their leaves. These results provide new insights into the role of miR7695 in regulating immune responses and Fe signaling pathways in the rice–*M. oryzae* interaction.

RESULTS

***M. oryzae* infection alters Fe distribution in rice leaves.**

In this work, we investigated the cellular distribution of Fe during *M. oryzae* infection by using the Perls staining. Perls reagent (potassium ferrocyanide) reacts with Fe^{3+} to form an insoluble pigment, or Prussian blue. Without pathogen infection, Perls staining revealed that Fe preferentially accumulated at stomata (**Fig. 1A upper left panel**). Upon pathogen challenge, iron staining showed a less uniform, but more widespread distribution in the stomatal areas pointing to a possible pathogen-induced iron mobilization (**Fig. 1A upper right panel**). Of interest, Perls staining revealed iron accumulation forming halo areas around the infection sites (**Fig. 1A, lower panels**).

To increase the sensitivity and definition of Fe detection in rice leaves, we used intensified Perls staining with DAB/H_2O_2. This method takes advantage of the redox activity of the Prussian blue reagent. As previously observed by Perls staining, after Perls/DAB staining, strong black precipitates appeared at stomatal areas in mock-inoculated leaves which showed a diffuse staining upon *M. oryzae* infection (**Fig. 1B, upper panels**). In those regions, Fe-stained granules were often visible (**Fig. 1B, middle left panel**). As well, in these regions, Fe accumulated with different intensities, with strong black precipitates in the center, surrounded by weaker and unevenly distributed halos of black precipitate (**Fig. 1B, middle right panel**). Higher magnification of these regions showed germinating spores and germ tubes forming appressoria, the sites where pathogen entry occurs (**Fig. 1B, lower panel**). Fe was weakly stained further away from the penetration site. Hence, histochemical analysis of Fe accumulation established that Fe accumulates at the sites of attempted penetration by the fungus (appressoria) as well as in cells in close proximity to the infection site, supporting that Fe distribution might be important for blast resistance.

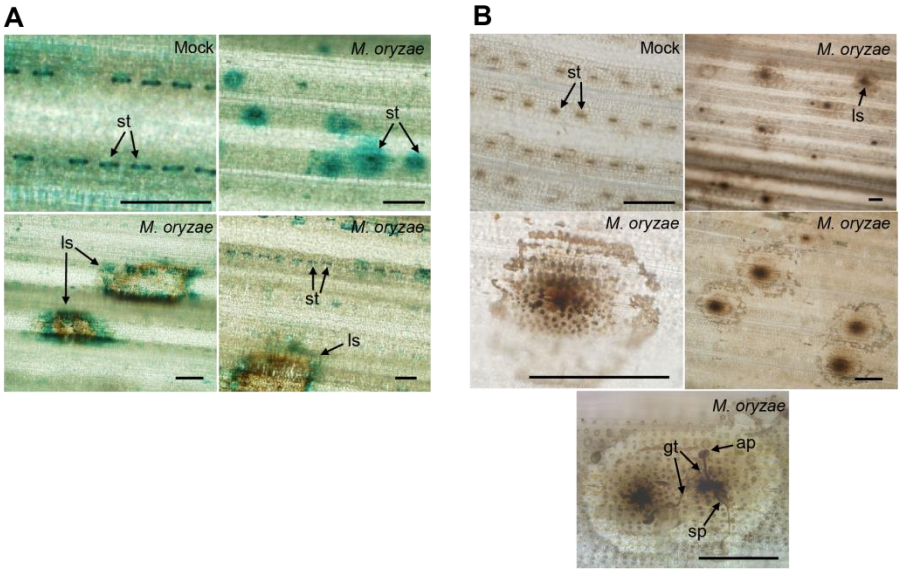


Figure 1 Histochemical detection of iron (Fe) in *M. oryzae*-infected rice (*O. sativa* cv. Nipponbare, *japonica*) leaves. Wild-type plants at the three-leaf stage were sprayed with a *M. oryzae* spore suspension or mock inoculated. At 24-48 hours post-inoculation (hpi), the third leaf of each plant was stained with Perls **(A)** or Perls/DAB **(B)** (blue and black precipitates, respectively). Scale bar: 100 µm. ap, appressorium; gt, germ tube; ls, lesion; st, stomata; sp, spore.

Resistance to infection by the rice blast fungus *M. oryzae* in mutant plants with *MIR7695* activation

We searched publicly available rice mutant collections for mutants with affected *MIR7695* expression. Because of the small size of *MIR* genes, identifying mutant alleles for miRNAs in insertional mutant collections is unlikely. A T-DNA tagged line (M0107013) was identified in the Taiwan Rice Insertion Mutants (TRIM) Database (Hsing *et al.* 2007); http://trim.sinica.edu.tw) in which the T-DNA was inserted upstream of the *MIR7695* locus (**Additional file 1: Fig. S1A, left panel**). TRIM was designed for gene knockout and activation tagging in the Tainung67 (*japonica*) background. Thus, the presence of an octamer of the *cauliflower mosaic virus 35S* (*CaMV35*) transcriptional enhancer next to the left border of the T-DNA can activate the expression of genes located up to 30 Kb from the integration site (Lo *et al.* 2016; Wu *et al.* 2017) (**Additional file 1: Fig. S1A, left panel**). Homozygous and azygous plants were identified by PCR genotyping (**Additional file 1: Fig. S1A, right panel,** primers are in **Additional file 2: Table S1**). Quantitative PCR (qPCR) revealed that *MIR7695*-Ac plants had a single copy of T-DNA inserted in its genome (**Additional file 3: Table S2**) Importantly, the accumulation of miR7695 precursor and mature sequences was higher in homozygous mutant plants with *MIR7695* activation tagging (hereafter *MIR7695*-Ac) than wild-type azygous (WT-Az) plants as revealed by RT-qPCR and small-RNA northern blot analyses, respectively (**Fig. 2A, left panel**). Consistent with upregulated *MIR7695*, the accumulation of miR7695 target transcripts (*OsNramp6.8*) was decreased in leaves with *MIR7695*-Ac (**Fig. 2A, right panel**).

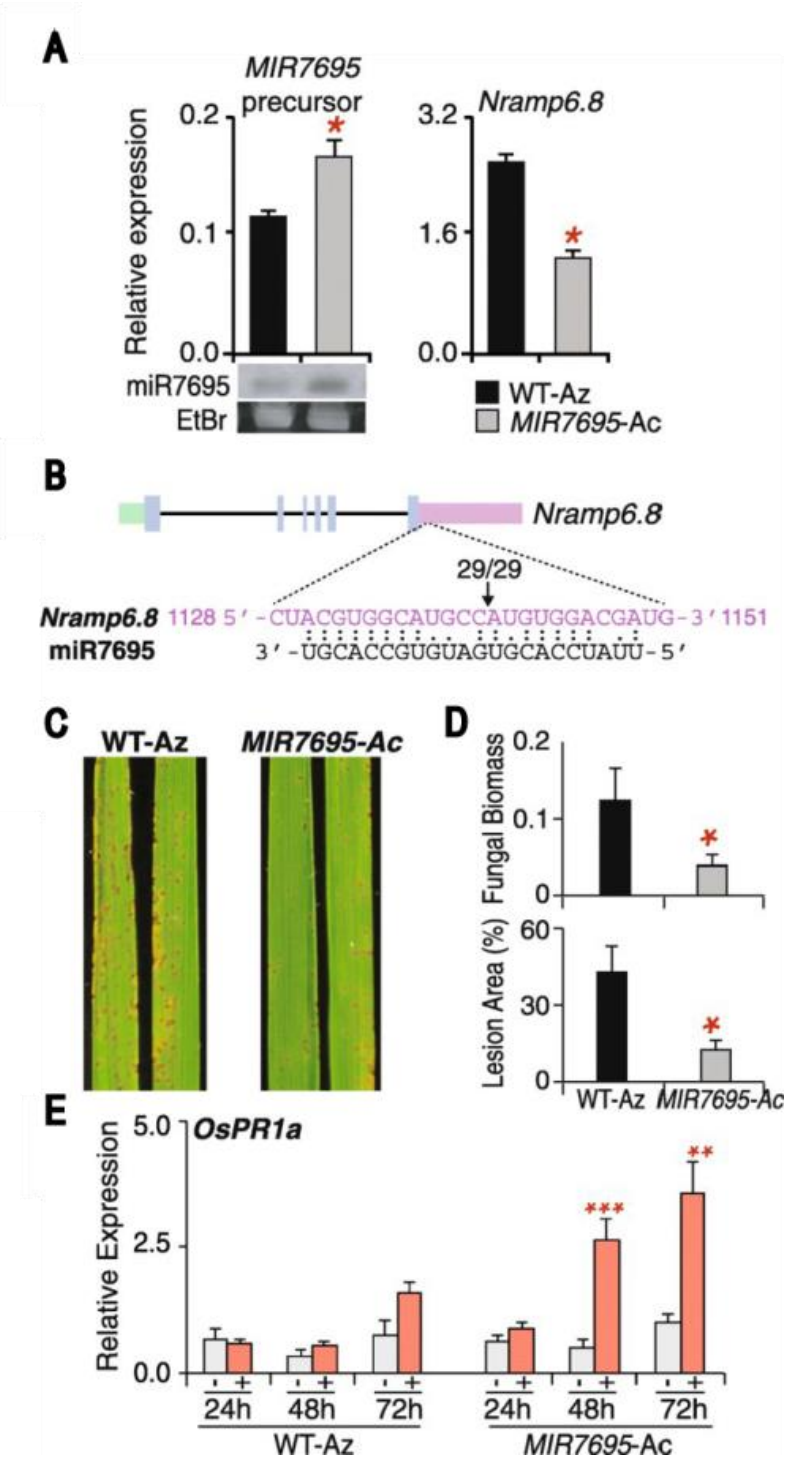

A
MIR7695
precursor
Relative expression
0.2
0.1
0.0
Nramp6.8
3.2
1.6
0.0
miR7695
EtBr
WT-Az
MIR7695-Ac
B
Nramp6.8
29/29
Nramp6.8 1128 5′-CUACGUGGCAUGCCAUGUGGACGAUG-3′1151
miR7695 3′-UGCACCGUGUAGUGCACCUAUU-5′
C
WT-Az
MIR7695-Ac
D
Fungal Biomass
0.2
0.1
0
Lesion Area (%)
60
30
0
WT-Az
MIR7695-Ac
E
OsPR1a
Relative Expression
5.0
2.5
0.0
24h
48h
72h
WT-Az
MIR7695-Ac

Figure 2 Resistance of *MIR7695-Ac* mutant plants to *M. oryzae* infection. **(A)** RT-qPCR analysis of *MIR7695* precursor transcripts (left panel) and miR7695 target (*Nramp6.8*, Os01g0503400.8) in homozygous mutant (*MIR7695*-Ac) and WT (segregated azygous, WT-Az) plants. Data are mean ±SE (n=3) (Student *t* test, *p<0.05). Lower panel: northern blot analysis of mature miR7695 using the miR7695.3-3p sequence as the hybridization probe (**Additional file 2: Table S1**). As a loading control, the RNA blot was stained with ethidium bromide (EtBr). **(B)** Experimental validation of miR7695-mediated cleavage of *OsNramp6.8* transcripts by 5'-RLM-RACE. Schematic representation of the *OsNramp6.8* (upper panel), showing the coding sequence (blue), 5'UTR (green), and 3'UTR (pink). Boxes, exons; lines, introns. Gene-specific primers were used for 5'-RACE and the resulting PCR products were sequenced. The identified cleavage site is indicated by an arrow and the number above indicate the detected cleavage site of independent clones. **(C)** Leaves of 3-week-old plants were sprayed with a *M. oryzae* spore suspension. The second leaf was photographed at 7 days post-inoculation. **(D)** Percentage of leaf area affected by blast lesions (upper panel). Relative fungal biomass (lower panel) was determined by qPCR as the ratio of *M. oryzae* 28S ribosomal DNA to the rice *Ubiquitin1* gene (primers in **Additional file 2: Table S1**). Data are mean ± SE (n=7) from 1 experiment (Student *t* test, *p<0.05). Four independent infection assays were performed with similar results. **(E)** RT-qPCR analysis of *OsPR1a* transcripts at different times after inoculation with *M. oryzae* spores. Blast infection was carried out as in (**C**). Data are mean ±SE (n=3, each biological replicate is a pool of 3 individual leaves) (Student *t* test, **p<0.01 ***p<0.001; infected *vs* non-infected). Mock inoculated (control) plants; +, *M. oryzae*-infected plants.

We previously reported that the recognition site of miR7695 locates in the 3' UTR region of *OsNramp6.8* transcripts. In this study, we further investigated whether *OsNramp6.8* gene is a real target gene for miR7695 by performing RNA ligase-mediated 5' RACE (5'-RLM-RACE). Sequencing of the 5' -RACE PCR products identified cleavage fragments at the expected site of *OsNramp6.8* transcripts, thus, supporting that *OsNramp6.8* transcripts are cleaved by miR7695 (**Fig. 2B**). These observations demonstrated that M0107013 is an activation mutant for *MIR7695* (*MIR7695*-Ac plants) and that miR7695 cleaves *OsNramp6.8* transcripts. *MIR7695*-Ac plants were slightly shorter and contained less chlorophyll than did WT-Az plants, but these differences were not statistically significant (**Additional file 1: Fig. S1B and C**).

Infection experiments were performed to assess the effect of *MIR7695* activation on disease resistance. WT-Az and *MIR7695*-Ac plants were spray-inoculated with *M. oryzae* spores. On visual inspection,

MIR7695-Ac plants were more resistant to *M. oryzae* infection than were WT plants (**Fig. 2C**). Blast resistance was confirmed by quantifying the lesion area and the relative amount of fungal DNA in infected leaves (**Fig. 2D**). Resistance of *MIR7695*-Ac plants to *M. oryzae* infection was also observed by local inoculation of detached rice leaves (**Additional file 4: Fig. S2**).

The induction of *PR1* expression is a widely used indicator of defense activation in response to pathogen infection in plants, including infection by *M. oryzae* in rice (Agrawal *et al.* 2001). As expected, *PR1a* was induced in WT-Az plants during *M. oryzae* infection (**Fig. 2E**). However, *PR1a* was induced at a much higher level in fungal-infected

MIR7695-Ac than WT-Az plants (**Fig. 2E**), which is consistent with the phenotype of blast resistance observed in *MIR7695*-Ac plants. The observed phenotype of blast resistance in *MIR7695*-Ac plants also agreed with resistance to *M. oryzae* infection in miR7695-overexpressing lines and *Osnramp6* mutant plants (Campo *et al.* 2013; Peris-peris *et al.* 2017).

As previously mentioned, without pathogen infection, iron accumulated in the stomata of leaves from wild-type rice plants whereas *M. oryzae* infection induced iron mobilization to the infection sites in wild-type plants (see **Fig. 1)**. In this work, we determined the accumulation of iron at different time points after inoculation with *M. oryzae* spores in wild-type and *MIR7695*-Ac plants. As it was observed in wild-type plants, iron was detected in stomata of *MIR7695*-Ac leaves in non-infected plants (**Fig. 3A)**. This analysis also revealed a stronger iron accumulation at the infection sites in the *MIR7695*-Ac plants compared to the WT-Az at 24 hpi (**Fig. 3B**, upper panels). Moreover, a general decrease on the iron content occurred at later time points (48 hpi, 72 hpi) in both wild-type and *MIR7695*-Ac plants.

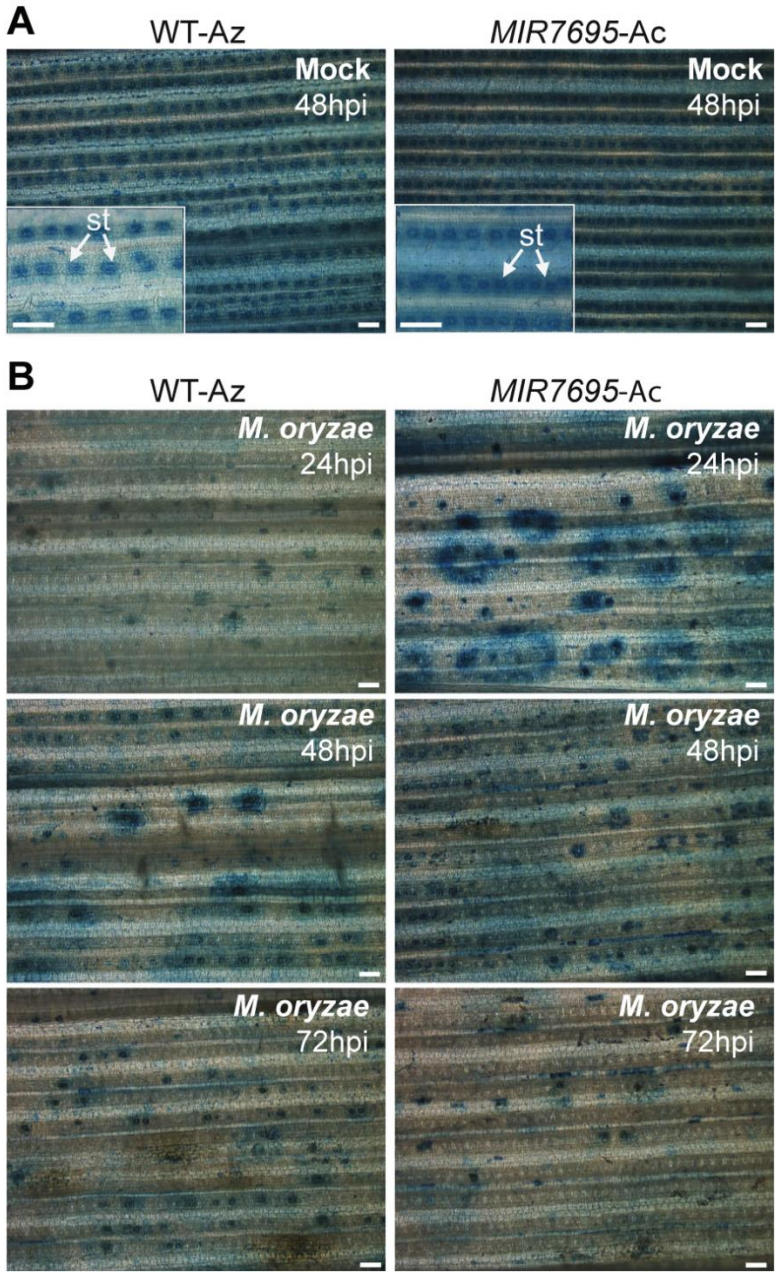


Figure 3 Histochemical detection of iron (Fe) in wild-type (*O. sativa* cv. Tainung 67, *japonica*) and *MIR7695*-Ac mutant plants during *M. oryzae* infection. Wild-type and *MIR7695*-Ac mutant plants at the three-leaf stage were (**A**) mock-inoculated or (**B**) inoculated with *M. oryzae* spores. At 24, 48, 72 hours post-inoculation (hpi), the third leaf of each plant was stained with Perls. Iron is detected as blue precipitates. Representative images of one experiment are shown (n=4). Three independent infection assays were performed with similar results. Scale bar: 100 µm. st, stomata.

Transcript profiling of *MIR7695*-Ac mutant plants

To investigate the molecular mechanisms underlying blast resistance in *MIR7695* plants, we used RNA-seq analysis. Initially, we examined the impact of *MIR7695* activation on the rice transcriptome by comparing the transcript profiles of mock-inoculated *MIR7695*-Ac and WT-Az plants. We identified 281 differentially expressed genes (DEGs; 153 upregulated and 128 downregulated) (**Fig. 4A; Additional file 5: Fig. S3A**). **Additional file 6: Table S3** lists the DEGs in *MIR7695*-Ac plants. Singular enrichment analysis (SEA) of molecular function by using AgriGO revealed gene ontology (GO) annotations in the "binding" and "catalytic activity" categories, which were over-represented for both upregulated and downregulated DEGs (**Fig. 4A; Additional file 7: Table S4**). Genes in the categories "transcription regulator activity" and "transporter activity" were specifically enriched in the upregulated DEGs, whereas genes in the "electron carrier activity" category were enriched in downregulated DEGs (**Fig. 4A**). The binding category comprised genes related to "calcium ion binding" and "zinc ion binding" (upregulated only in *MIR7695*-Ac plants) and "iron ion binding" genes (downregulated only in *MIR7695*-Ac plants) (**Fig. 4B**).

The expression of a vast array of transcription factors (TFs) belonging to different TF families was regulated in mock-inoculated *MIR7695*-Ac plants (most of them being upregulated) (**Fig. 4C; Additional file 8: Table S5**). They included TFs with a demonstrated role in the rice defense response to blast infection), such as *OsWRKY45* and *OsNAC4* (Wei *et al.* 2013). These TFs function as positive regulators of the rice response to *M. oryzae* infection (Kaneda *et al.* 2009; Shimono *et al.* 2012) and are both upregulated in *MIR7695-Ac* plants (**Fig. 4C**). Other TFs that are activated in *MIR7695* plants are known to mediate defense hormone signaling, such as ethylene response factor 5 (*OsEREBP5*), APETALA2/ethylene-responsive element binding protein (AP2/EREBP), several jasmonate ZIM-domain (JAZ) TFs, and *RERJ1* (a jasmonic acid-dependent stress inductive bHLH transcription factor)

(Miyamoto *et al.* 2013) (**Fig. 4C**). Genes encoding several wall-associated kinase (WAK) receptors and disease resistance (R) proteins were also upregulated in *MIR7695*-Ac (**Fig. 4C**). Upregulated genes in *MIR7695-Ac* plants also included several heavy metal transporter/metal detoxification (*HMTD*) protein genes and siroheme uroporphyrinogen methyltransferase1 (*SUM1*), encoding enzymes responsible for the synthesis of the Fe-containing cofactor of enzymes (**Fig. 4C**).

Genes that were downregulated in mock-inoculated *MIR7695-Ac* plants included those involved in the synthesis of nicotinamine (NA), a chelator of metals and the precursor of phytosiderophores (components for Fe acquisition) (Inoue *et al.* 2003): *OsNAS1* and *OsNAS2*, encoding nicotinamine synthases (**Fig. 4C).** Other downregulated genes are involved in oxidation-reduction processes, such as laccases (*OsLAC4*, *OsLAC19, OsLAC24*) and peroxidases (*Prx81, Prx125*) (**Fig. 4C**).

RT-qPCR was used to validate RNA-seq findings. RT-qPCR results obtained for selected genes were highly concordant with RNA-seq results for both upregulated genes (*OsWRKY45, OsWRKY71, OsNAC4, OsDREB1G*, *OsDREB1E*, *OsRERJ1*) and downregulated genes (*OsLAC19* and *OsNAS1*) (**Additional file 9: Fig. S4**).

Together, these observations suggest that without pathogen infection, *MIR7695* activation led to altered expression of genes involved in 1) transcriptional regulation, 2) disease resistance, 3) metal binding and transport, and 4) oxidation-reduction mechanisms. Transcriptional changes caused by *MIR7695* activation might well contribute to the resistance response of these plants to pathogen infection.

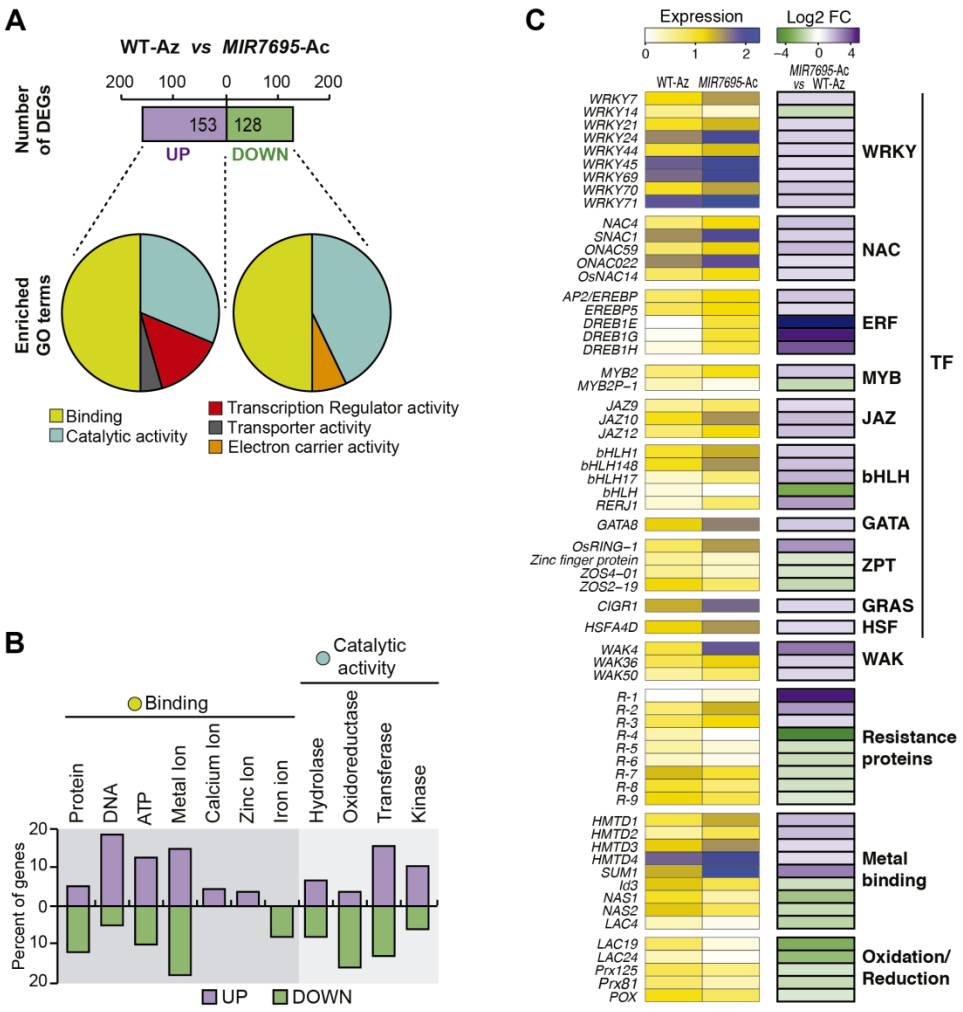


Figure 4 Differentially expressed genes (DEGs) in *MIR7695*-Ac mutant plants relative to WT-Az plants by RNA-seq analysis. Leaves of three-week-old plants were used **(A)** Number of DEGs and Gene Ontology (GO) analysis of DEG function. Up-regulated genes (log2 fold change [FC] ≥ 1; purple) and down-regulated genes (log2FC ≤ -1; green) genes (p <0.05, false discovery rate [FDR] <0.05, n=2). Pie charts represent the five general GO terms enriched in up- and downregulated DEGs. **(B)** Enriched terms in the "binding" and "catalytic activity" categories. **(C)** Heatmaps showing RNAseq expression level (left panel; log10 [FPKM+1]) and FC (right panel; log2FC) of DEGs. Gene expression is represented from pale yellow (less expressed) to blue (more expressed). Upregulated (log2FC ≥ 1; purple) and downregulated (log2FC ≤ -1; green) DEGs. Data are means (n=2). The full gene names and ID list are in **Additional file 8: Table S5**.

Enhanced defense responses to *M. oryzae* infection in *MIR7695*-Ac plants

Pathogen-induced alterations in the transcriptome of *MIR7695*-Ac plants were identified and compared to those of fungal-infected WT-Az plants. The number of genes with expression affected by *M. oryzae* infection at 48 hours post-infection (hpi) was 4.5 times higher in *MIR7695*-Ac than WT-Az plants (531 and 116, respectively) (**Fig 5A; Additional file 5: S3bc**). This observation already indicated stronger transcriptional regulation in the mutant plants. DEGs for WT-Az and *MIR7695*-Ac plants are listed in **Additional file 10 and 11 (Tables S6** and **S7),** respectively. Of note, genes typically associated with the plant response to pathogen attack, such as *PR* genes, were induced in *MIR7695-Ac* but not in WT-Az plants at 48hpi (**Fig. 5B; Additional file 12: Table S8**). They included *PR1*, *β-1,3-glucanase* (*PR2*), *chitinase* (*PR3*, *PR4*, *PR8*), *thaumatin* (*PR5*), *peroxidase* (*PR9*), *PBZ1* and other *Bet v1* homologues (*PR10*), and *lipid transfer protein* (*LTP*; *PR14*). The antimicrobial activity of many of these PR proteins has been demonstrated (e.g., PR1, chitinases, β-1,3-glucanases, PR4, thaumatin, LTPs) (Ali *et al.* 2018).

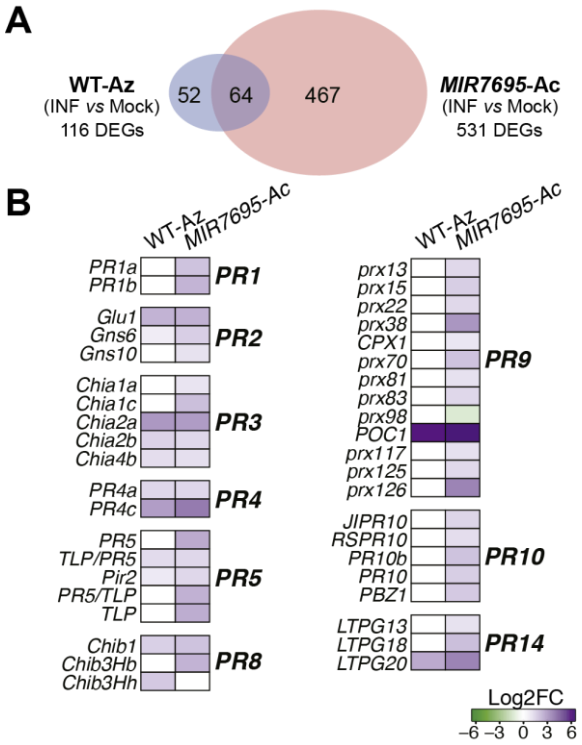


Figure 5 Comparison of DEGs in *MIR7695*-Ac and WT plants after challenge with *M. oryzae*. Leaves of 3-week-old rice plants (WT-Az and *MIR7695*-Ac) were mock-inoculated or sprayed with a suspension of *M. oryzae* spores, and collected at 48 hpi for RNA extraction and RNA-seq. Upregulated (log2FC ≥ 1) and downregulated (log2FC ≤ 1) genes by *M. oryzae* infection ($p < 0.05$, FDR <0.05). **(A)** Venn diagram of the overlap between fungal-responsive genes of each genotype. **(B)** Comparison of the expression pattern of defense-related genes with *M. oryzae* infection. Up- (purple) and downregulated (green) DEGs. For a full list of gene IDs, see **Additional file 12: Table S8**.

To further establish differences in the transcriptional response to pathogen infection between *MIR7695-Ac* and WT-Az plants, we used a two-factor analysis (genotype and treatment) of the full dataset of DEGs in each genotype. A total of 153 and 100 genes were identified as upregulated and downregulated, respectively, in *MIR7695*-Ac versus WT plants (**Fig. 6A** and **S3D; Additional file 13: Table S9**). AgriGO was used for GO enrichment analysis of DEGs in the bifactorial analysis, with clustering by Revigo (**Fig. 6A**). The GO terms over-represented in upregulated genes were clustered in the categories "response to stress" (biotic and oxidative stress), "response to stimulus", and "secondary metabolism" (phenylpropanoids and terpenoids) (**Fig. 6A, upper panel**). Genes induced in *MIR7695*-Ac plants in the bifactorial analysis included defense-related genes, such as *PR* genes (*PR1*, *PR2*, *PR5* and *PR10* family members) and oxidative stress-related enzymes (e.g., several peroxidases) (**Fig. 6B** and **Additional file 14: Table S10**).

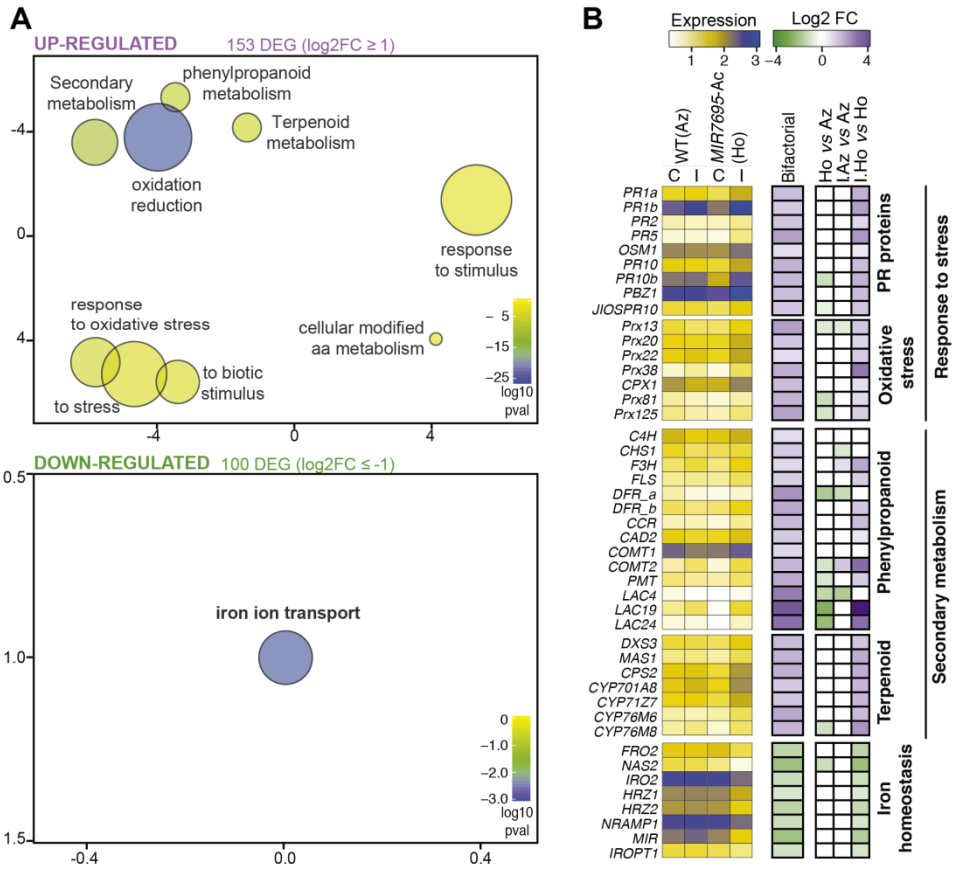


Figure. 6 Biological processes altered in *MIR7695*-Ac mutant plants relative to WT-Az plants with *M. oryzae* infection. Same plant material as in Fig. 4. RNA-seq data underwent bifactorial analysis (upregulated, log2FC ≥ 1; downregulated, log2FC ≤ 1; p <0.05, FDR <0.05). **(A)** GO analysis of DEG function in *MIR7695*-Ac plants with blast infection (upper and lower panels show upregulated and downregulated DEGs, respectively). The top GO terms enriched in *MIR7695*-Ac *vs* WT-Az DEGs were represented by using REVIGO after reducing redundancy (http://revigo.irb.hr/). Circles represent GO terms and those clustered closer to each other represented similar GO terms. Disc colors (blue to yellow) represent de degree of GO enrichment (p-value) and disc size is proportional to the frequency of the GO term in the GO database (larger and smaller discs represent more general and more specific terms, respectively). **(B)** Heatmap showing distribution of RNAseq expression level (log10[FPKM+1], pale yellow to blue from less to more expressed) for DEGs belonging to the top enriched GO categories in *M. oryzae*-infected *MIR7695*-Ac plants (left panel). Heatmaps show upregulated (purple) and downregulated (green) DEGs (bifactorial analysis, middle panel; Monofactorial analysis for the given comparisons, right panel) Biological processes are indicated to the left. Data are means (n=2). The full gene ID list is shown in **Additional file 14: Table S10.**

Phenylpropanoid biosynthetic genes were highly represented in the bifactorial analysis of DEGs. They included genes involved in the production of flavonoids (*CHS; F3H, FLS, DFR*) and monolignols, the building blocks of lignin (*CCR, CAD, COMT, PMT, LAC, PRX*) (**Fig. 6B** and **Additional file 14: Table S10**; additional information on phenylpropanoid and lignin biosynthesis genes that were differentially regulated in infected *MIR7695*-Ac plants is in **Additional file 15: Fig. S5A)**. The expression of flavonoid biosynthesis genes is known to be induced by pathogen infection, and certain plant flavonoids exhibited antifungal activity (Galeotti *et al.* 2008). The accumulation of lignin in secondary cell walls provides a physical barrier against pathogen invasion (Miedes *et al.* 2014). The expression of several peroxidases was upregulated in *MIR7695-Ac* versus WT-Az plants and also with pathogen infection. Peroxidases are key enzymes in the biosynthesis of lignin during resistance reactions via cross-linking of lignin monomers. A stronger induction of genes involved in flavonoid and lignin biosynthesis might play a role in protecting the *MIR7695*-Ac plants against *M. oryzae* infection. In addition, a important number of genes involved in the production of diterpenoid phytoalexins were among the top induced genes in *MIR7695*-Ac plants with infection (bifactorial DEGs) (**Fig. 6B; Additional file 14: Table S10**) as described below.

The GO term most represented in downregulated genes on bifactorial analysis of DEGs was “iron ion transport” (**Fig. 6A, lower panel**). This included genes related to Fe homeostasis, such *OsFRO2* (a Fe^{3+} reductase), *OsNAS2* (a nicotianamine synthase), *OsIRO2* TF, *OsHRZ1, OsHRZ2* ubiquitin ligases, *OsNRAMP1* (Fe^{2+} transporter), *OsMIR* (mitochondrial Fe-regulated gene), and *OsIROPT* (an oligopeptide transporter) (**Fig. 6B; Additional file 14: Table S10**).

A more detailed expression analysis was performed for genes identified by bifactorial analysis, and their expression was examined by RT-qPCR at different times after inoculation with *M. oryzae* spores (24, 48 and 72 hpi). This analysis confirmed stronger induction of *PR* genes (*OsPR1b, OsPBZ, OsPR10b*) and lignin biosynthesis genes (*OsCAD2,*

OsCOMT1) in *MIR7695*-Ac than WT-Az plants during *M. oryzae* infection (**Fig. 7**). Induction of *OsPBZ1* and other *OsPR10* family members is known to occur during *M. oryzae* infection and, when overexpressed, the genes confer pathogen resistance (Huang *et al.* 2016; Kawahara *et al.* 2012; Wang *et al.* 2014).

Altogether, comparative transcriptome analysis (bifactorial analysis) revealed stronger induction of defense-related genes in *MIR7695*-Ac (e.g., *PR*, oxidative stress-related, phenylpropanoid and diterpenoid phytoalexin biosynthesis genes), whereas genes that function in Fe homeostasis appear to be downregulated in *MIR7695-Ac* plants during *M. oryzae* infection.

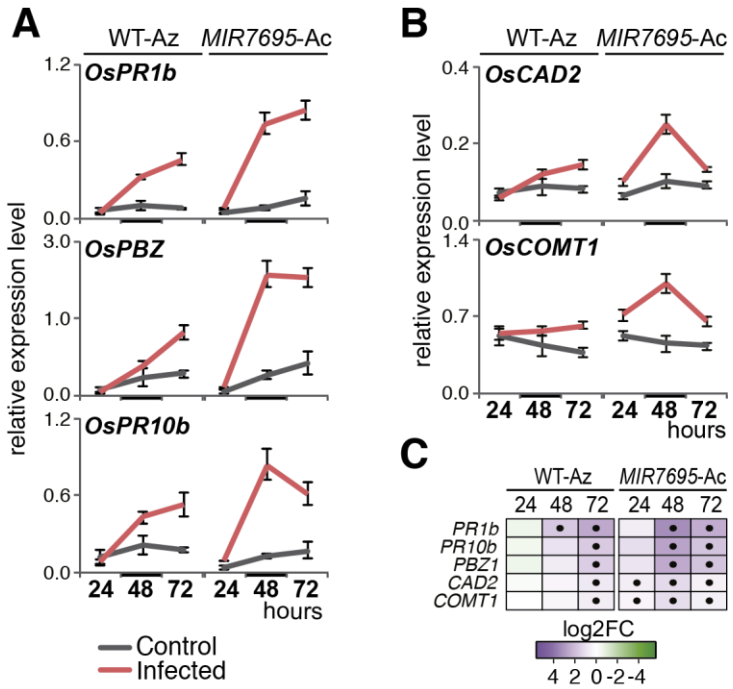


Figure. 7 Expression of *PR* and lignin biosynthesis genes in WT-Az and *MIR7695*-Ac plants during blast infection. Plants were sprayed with a *M. oryzae* spore suspension. Leaves were collected at the indicated times (24, 48, 72 hpi). **(A-B)** Expression analysis of **(A)** *PR* (*OsPR1b, OsPBZ, OsPR10b*) and **(B)** lignin (*OsCAD2, OsCOMT1*) genes determined by RT-qPCR. Data are mean ± SE (n=3; each sample consisted of a pool of 3 individual leaves). Mock-inoculated (control, grey) and *M. oryzae*-infected (red) plants. Time point used for RNAseq analysis (48 h) is labeled with a thick line in the x-axis. **(C)** Heatmap showing log2 FC for each transcript and each time (infected *vs.* control) as determined from RT-qPCR values (a-b). Upregulated (purple) and downregulated (green). Dots indicate significant differences (infected vs control) (Student *t* test, $p < 0.05$).

Regulation of Fe homeostasis-related genes in rice leaves during *M. oryzae* infection

In plant roots, two different mechanisms have been described for Fe uptake from the rhizosphere, the reducing and chelating strategies (strategies I and II, respectively) (Kobayashi and Nishizawa 2012; Pereira *et al.* 2014). Rice is unique in that as it uses both strategies. Besides strategy I and II genes, other genes contribute to Fe transport and/or mobilization through the plant. Although great progress has been made during the last years to identify mechanisms governing Fe uptake in roots, the regulation of Fe homeostasis genes in leaves is less understood.

We investigated the expression profile of Fe homeostasis-related genes in leaves of wild-type plants during *M. oryzae* infection. Genes examined were: *OsFRO2, OsIRO2, OsHRZ1, OsNRAMP1* and *OsIROPT1* (genes strongly downregulated in *MIR7695*-Ac plants). These genes were strongly upregulated early during infection (24-48 hpi) but downregulated at a later stage of the infection process (72 hpi) (**Additional file 16: Fig. S6;** WT-Az, infected *vs* mock). Upregulation of these genes early during infection of WT plants correlates with Fe accumulation at the sites of fungal penetration and infection sites, as revealed by histochemical analysis of *M. oryzae*-infected rice leaves (**Fig. 1**).

For a comparison, we examined the expression profile of Fe homeostasis genes in *MIR7695*-Ac plants. Four of the five genes examined were induced early during infection (24 hpi), as it was observed in WT plants, followed by a strong downregulation at 48 and 72 hpi (**Additional file 16: Fig. S6;** *MIR7695-Ac*, infected *vs* mock). Therefore, downregulation of Fe homeostasis genes occurs earlier in *MIR7695-Ac* than WT-Az plants.

Phytoalexins accumulate in *MIR7695*-Ac plants during *M. oryzae* infection

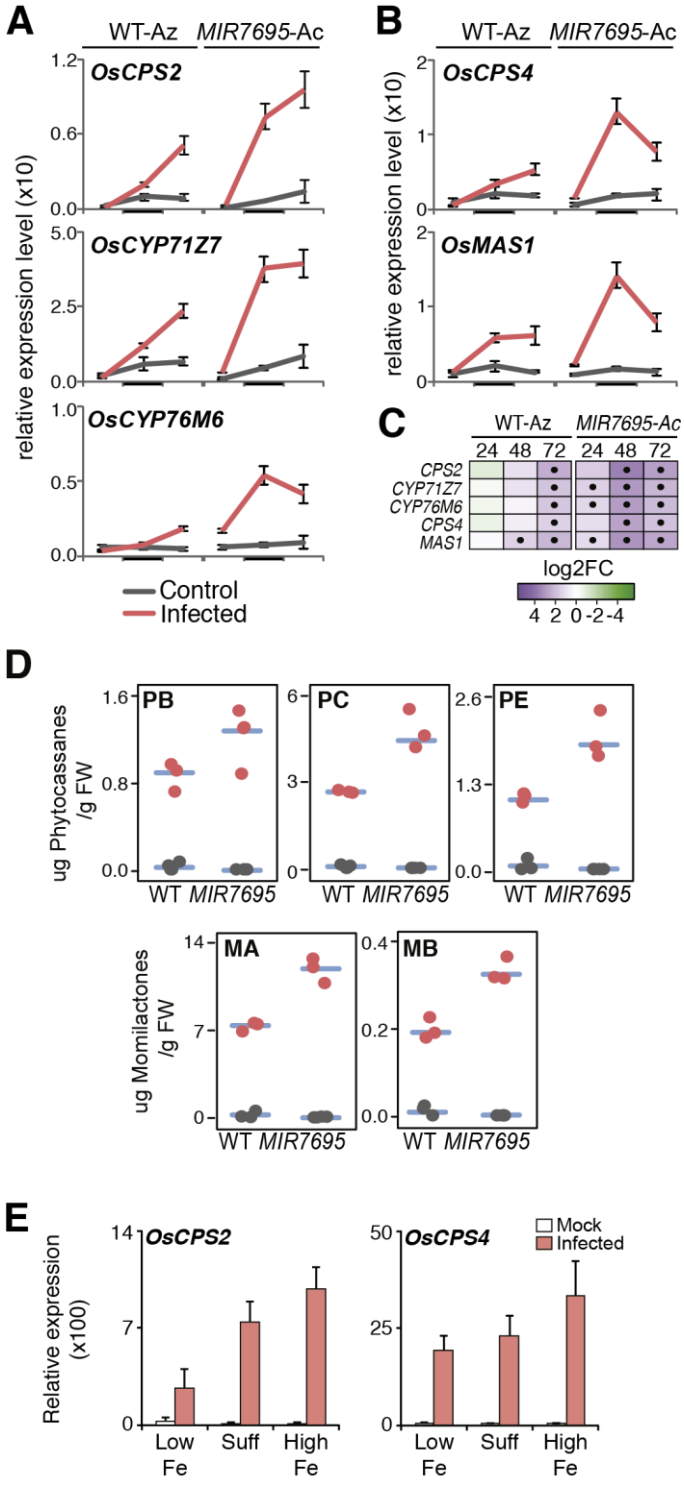
Phytoalexins are low-molecular-weight antimicrobial compounds that accumulate in plant tissues during pathogen infection (Ahuja *et al.* 2012). Major phytoalexins accumulating in rice leaves in response to *M. oryzae* infection are the diterpene phytoalexins momilactones, phytocasssenes and oryzalexins (Hasegawa *et al.* 2010). As previously mentioned, the expression of genes involved in the biosynthesis of diterpenoid phytoalexins, oryzalexins, phytocassenes and momilactones was induced to a higher level in *MIR7695-Ac* than WT-Az plants (at 48 hpi with *M. oryzae*) (see **Fig. 6B)**. For details on genes involved in diterpene phytoalexin biosynthesis with overexpression in *MIR7695* plants, see **Additional file 15: Fig. S5B**. RT-qPCR analysis of diterpene phytoalexin biosynthesis genes at different times after inoculation with *M. oryzae* spores (24, 48, 72 hpi) confirmed earlier and stronger induction of these genes in *MIR7695*-Ac than WT-Az plants (**Fig. 8A, B**). Differences in pathogen-induced expression of these genes were more evident at 48 and 72 hours after blast inoculation, as revealed by the higher fold change of gene expression (**Fig. 8C**).

To investigate whether superinduction of diterpenoid phytoalexin biosynthesis genes affects phytoalexin accumulation, we measured phytocassane and momilactone levels in leaves of *MIR7695*-Ac and WT-Az plants without and with infection. As expected, the expression of phytocassanes (B, C and E) and momilactones (A and B) was barely detected in non-infected rice leaves (**Fig. 8D**). Upon pathogen challenge, the accumulation of phytocassanes and momilactones increased in both WT-Az and *MIR7695-Ac* plants but was significantly higher in *MIR7695*-Ac than WT plants (**Fig. 8D**). These findings revealed that in response to pathogen infection, diterpenoid phytoalexin biosynthesis genes are induced earlier in *MIR7695*-Ac than WT-Az plants. *MIR7695*-Ac plants also accumulated higher levels of phytoalexins during pathogen infection. Knowing that diterpene phytoalexins have antifungal activity against *M.*

oryzae (Dillon *et al.* 1997; Hasegawa *et al.* 2010; Umemura *et al.* 2003), a higher *M. oryzae*-induced accumulation of phytoalexins in leaves of *MIR7695-Ac* plants might contribute to disease resistance in these plants.

Finally, we investigated whether Fe supply affects the expression of genes involved in the biosynthesis of diterpenoid phytoalexins in rice. We analyzed the effect of Fe supply (low, sufficient and high) on the expression of *OsCPS2* and *OsCPS4*, which function first cyclization steps in the phytoalexin biosynthetic pathway (**Additional file 15: Fig S5B**). The expression of these genes was barely detected in plants without infection (**Fig. 8E**). Upon pathogen challenge, the highest expression of phytoalexin genes occurred in plants grown under high Fe supply as compared with low or sufficient Fe (**Fig. 8E**), which supports that Fe supply affects phytoalexin biosynthesis. Presumably, a localized accumulation of Fe at the sites of pathogen penetration and/or invasion would activate the expression of phytoalexin biosynthetic genes for phytoalexin accumulation, thus arresting fungal colonization in infected leaves of *MIR7695*-Ac plants.

Figure. 8 Expression of diterpenoid phytoalexin genes in WT-Az and *MIR7695*-Ac plants during blast infection. Plant material was treated as in Fig. 7. **(A-B)** RT-qPCR analysis of expression of **(A)** Phytocassane (*OsCPS2*, *OsCYP71Z7*, *OsCYP76M6*) and **(B)** momilactone (*OsCPS4*, *OsMAS1*) biosynthesis genes in rice leaves infected with *M. oryzae*. Data are mean ± SE (n=3; each sample consisted of a pool of 3 individual leaves). Mock-inoculated (control, grey) and *M. oryzae*-infected (red) plants. **(C)** Heatmap showing log2 FC for each transcript and each time point (infected *vs* control) as determined from RT-qPCR values (a-b). Upregulated (purple) and downregulated (green). Dots indicate significant differences (infected *vs* control) (Student *t* test, $p < 0.05$). **(D)** Accumulation of diterpenoid phytoalexins, phytocassane E (PE), B (PB) and C (PC) (upper panels) and momilactone A (MA) and B (MB) (lower panels), in leaves of mock- and *M. oryzae*-infected plants. Each dot represents a biological replicate. FW, fresh weight. **(E)** RT-qPCR of expression of upstream diterpenoid biosynthetic genes (*OsCPS2* and *OsCPS4*) in mock- and *M. oryzae*-infected leaves of rice plants treated under three different Fe supply conditions (low, sufficient, high). Data are mean ± SE (n=3), each sample consisting of a pool of 4 individual leaves).


A
WT-Az
MIR7695-Ac
OsCPS2
OsCYP71Z7
OsCYP76M6
relative expression level (x10)
Control
Infected
B
OsCPS4
OsMAS1
C
24 48 72 24 48 72
CPS2
CYP71Z7
CYP76M6
CPS4
MAS1
log2FC
4 2 0 -2 -4
D
PB
PC
PE
MA
MB
ug Phytocassanes /g FW
ug Momilactones /g FW
WT MIR7695
E
OsCPS2
OsCPS4
Relative expression (x100)
Mock
Infected
Low Fe
Suff
High Fe

DISCUSSION

Although an increasing number of miRNAs have been shown to be differentially expressed in response to pathogen infection or nutrient stress, most of this research involved plants exposed to one or another type of stress separately. Furthermore, few studies aimed to understand the regulation of Fe homeostasis in rice during *M. oryzae* infection. Here, we present evidence of a miR7695-guided cleavage of *OsNramp6.8* transcripts encoding the NRAMP6 iron transporter from rice. Moreover, we investigated the role of miR7695 in the rice response to infection by *M. oryzae*. Upon challenge with *M. oryzae*, Fe accumulated near *M. oryzae* appressoria and in cells surrounding infected regions of rice leaf. Very recently, Dangol *et al.* reported that incompatible rice/*M.oryzae* interactions trigger iron- and ROS-dependent ferroptotic cell death in leaf sheaths of rice plants where iron accumulated at sites of infection to mediate the oxidative burst (Dangol *et al.* 2019). Activation-tagged *MIR7695* rice plants showed enhanced resistance and a stronger accumulation of iron at the sites of infection. On RNA-seq analysis, defense-related genes, including *PR* and diterpenoid biosynthetic genes were strongly induced along with blast resistance in *MIR7695*-Ac plants. Levels of phytoalexins during pathogen infection were higher in *MIR7695*-Ac than WT azygous plants and genes in the phytoalexin biosynthetic pathway were highly induced in rice plants grown under high Fe supply. This piece of evidence support that miR7695 positively regulates immune responses and establish links between defense signaling and Fe homeostasis in rice. However, the exact mechanisms by which Fe signaling regulates the expression of defense-related genes remains to be determined.

Being a foliar pathogen, *M. oryzae* has an absolute requirement for Fe from host tissues, so rice plants might capitalize on the toxicity or the essentiality of Fe to arrest *M. oryzae* invasion. Different scenarios can be considered. On the one hand, mechanisms that exploit Fe toxicity might be used by the host plant against *M. oryzae*. On the other, the host plant

might develop withholding strategies to restrict Fe availability to the invading pathogen, a process that in humans and animals has been called "nutritional immunity" (Hood and Skaar 2012). An examination of Fe distribution in *M. oryzae*-infected WT rice leaves revealed Fe accumulation in close vicinity of appressoria and in cells surrounding the infection sites, thus, reinforcing the notion that rice plants use strategies to locally increase Fe levels to prevent penetration and spread of the pathogen into the leaf tissue. Local accumulation of Fe would avoid Fe poisoning caused by a generalized accumulation of Fe in rice leaf while providing a signal for the activation of host immune responses. If so, this localized accumulation at the sites of pathogen penetration and invasion might mediate a localized oxidative burst that can be toxic to the invading pathogen. Local accumulation of H_2O_2 would also serve for cell-wall reinforcement (lignification, oxidative cross-linking of cell wall components) and induction of defense-related genes (e.g., *PR* genes).

Without infection, Fe preferentially accumulated in leaf stomata. In this respect, Fe has been shown to be important in regulating aperture of stomata (Gusmão *et al.* 2013). During *M. oryzae* infection, a re-distribution of Fe appears to occur in the rice leaf, Fe moving around stomata and toward the sites of pathogen penetration and colonization. In support of this notion, a localized accumulation of Fe in cell wall appositions and subsequent defensive H_2O_2 production was previously linked to basal defense in wheat leaves after infection with *Blumeria graminis* f. sp. *tritici* (Liu *et al.* 2007). Also, altered Fe distribution in Arabidopsis plants infected with the bacterial pathogen *Dickeya dadantii* was reported (Aznar *et al.* 2015). Although not proven, the activation of toxic oxidative bursts caused by localized accumulation of Fe in rice leaves might be important to restrict *M. oryzae* growth while maintaining normal plant development. Iron accumulation at the sites of pathogen infection was observed in both wild-type and *MIR7695-Ac* plants, the later ones accumulating more iron at the infection sites than wild-type plants.

During *M. oryzae* infection, genes involved in Fe homeostasis were strongly downregulated in leaves of *MIR7695-Ac* plants compared with WT plants. As previously mentioned, the rice plant uses a combined strategy for Fe uptake from the rhizosphere that has features of both strategy I (reduction of Fe^{3+} to Fe^{2+}, a system that operates in roots of most non-graminaceous species) and strategy II (release of phytosiderophores by the root, typical of graminaceous species) (Ricachenevsky and Sperotto 2014). Our results indicate that during *M. oryzae* infection, genes that function in Fe uptake via strategy I (e.g. *OsFRO2*) or strategy II (e.g. *OsIRO2*, *OsNAS2*) in roots are downregulated in leaves in both WT-Az and *MIR7695*-Ac plants. Other Fe homeostasis genes such as *NRAMP1* (a Fe transporter), *OsHRZ1* and *OsIROPT1* are also downregulated during infection. In line with this, the Fe homeostasis genes *TmFER1* and *TmNAS1* (marker genes for monitoring intracellular Fe status in wheat) were found downregulated in *B. graminis*-infected wheat leaves (Liu *et al.* 2007). Furthermore, downregulation of Fe homeostasis genes was accompanied by cytosolic Fe depletion and induction of *PR* genes. A better understanding of the mechanisms involved in Fe homeostasis in rice leaf tissues is needed to know whether *M. oryzae* also provokes intracellular Fe depletion in rice leaves.

MIR7695-Ac plants exhibited resistance to *M. oryzae* infection, which is consistent with the phenotype of disease resistance observed in loss-of-function *OsNramp6* plants (Peris-peris *et al.* 2017). Disease resistance in *MIR7695*-Ac plants is associated with a basal expression of resistance genes and defense regulatory genes (e.g., *OsWRKY45*, *OsNAC4*) without pathogen infection and a superinduction of defense-related genes with infection. Thus, *MIR7695*-Ac plants mount a stronger defense response to pathogen infection, a response that is reminiscent of defense priming (Conrath *et al.* 2015). Whether defense responses are activated earlier in *MIR7695*-Ac than WT plants is unknown. Furthermore, proteins encoded by defense-related genes that are strongly induced during infection in *MIR7695*-Ac plants are known to possess

antimicrobial activity (e.g., chitinases, β-1,3-glucanases, PR10 and LTP proteins), and their overexpression in plants confers pathogen resistance, including blast resistance (Huang *et al.* 2016; Selitrennikoff 2001). Stronger expression and induction of peroxidases is also a feature of *MIR7695*-Ac plants, these genes being typically induced in host plant tissues upon pathogen infection. Peroxidases are important for generating highly toxic environments by producing ROS species during resistance reactions (Almagro *et al.* 2009) and for lignin biosynthesis (cross-linking of lignin monomers). A miR7695-mediated regulation of peroxidases might then function to generate an oxidative burst at the sites where Fe accumulates, thus helping to limit pathogen spread on the rice leaf. Also, an important number of genes involved in the flavonoid and lignin branches of the general phenylpropanoid pathway were upregulated in *MIR7695-Ac* versus WT plants (bifactorial analysis). The antifungal activity of phenylpropanoid compounds against phytopathogens has been reported (Camargo-Ramírez *et al.* 2018; Cho and Lee 2015; Dixon *et al.* 2002). The superactivation of these various defense genes might be responsible for the blast resistance phenotype observed in *MIR7695*-Ac plants.

Notably, upon pathogen challenge, diterpenoid phytoalexin biosynthesis genes were highly upregulated in *MIR7695*-Ac plants (bifactorial analysis), accompanied by increased accumulation of major rice phytoalexins. For some of these phytoalexins, antifungal activity against *M. oryzae* has been described (Dillon *et al.* 1997; Hasegawa *et al.* 2010; Umemura *et al.* 2003). Other studies proposed that rapid biosynthesis of diterpene phytoalexins contributes to resistance to *M. oryzae*, whereas delayed induction of these genes results in enhanced susceptibility to blast infection (Hasegawa *et al.* 2010). The accumulation of phytoalexins would enhance the ability to cope with pathogen infection in *MIR7695*-Ac plants.

CONCLUSIONS

Overall, this study highlights the relevance of miR7695 in blast resistance via regulation of rice immune responses. Because miR7695 regulates *OsNramp6* encoding a Fe transporter from rice, these results support the existence of links between miR7695/*OsNramp6* functioning to control Fe signaling and defense signaling in rice. At the cellular level, *M. oryzae* infection altered Fe distribution in rice leaves, a process probably involving miR7695. Because miRNAs function as fine-tuners of gene expression instead of turning-on or turning-off target gene expression, miR7695 would be well suited to maintain appropriate Fe levels in host cells during pathogen infection. If so, miR7695 might well be involved in modulation of iron accumulation in tissues of the rice leaf which, in turn, would affect the expression of Fe homeostasis genes. The current challenge of basic and applied plant research is to understand interconnected regulations between miR7695-mediated mechanisms involved in Fe homeostasis and disease resistance in plants. Deciphering the mechanisms involved in Fe distribution and remobilization during *M. oryzae* infection with the participation of miR7695 will help in designing innovative strategies for blast disease control. Knowing how plants integrate immune responses and Fe signaling pathways is an issue of great importance in both basic and applied plant research.

MATERIALS AND METHODS

Plant material, growth conditions and genotyping

Rice plants were grown at 28ºC with a 14 h/10 h light/dark cycle. The T-DNA insertion line for *MIR7695* (*O. sativa* cv. Tainung67, *japonica*) was obtained from the Taiwan Rice Insertion Mutant (TRIM) collection from the Academia Sinica of Taiwan (Hsing *et al.* 2007); http://trim.sinica.edu.tw), and propagated under controlled conditions (CRAG greenhouse Service). For genotyping, genomic DNA was extracted as described (Murray and Thompson 1980) but with mixed alkyltri-methylammoniumbromide (MATAB) used as the extraction buffer (0.1 M Tris–HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% MATAB, 1% PEG 6000, 0.5% sodium sulphite). PCR genotyping (100 ng DNA/PCR reaction) involved specific primers (*P1* and *P3*) and T-DNA–specific primers (*P2*) (**Additional file 2: Table S1**). T-DNA copy number was estimated as described (Yang *et al.* 2005).

For Fe treatment, 10 rice seeds were grown in 0.35-L pots containing soil (turface: vermiculite:quartz sand [2:1:3]) for 14 days and then watered with a half-strength Hoagland solution (5 mM KNO_3, 5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2 mM $MgSO_4 \cdot 7H_2O$, 1 mM NH_4NO_3, 0.5 mM 1 M KH_2PO_4 (pH to 6.0), 46.3 µM H_3BO_3, 9.1 µM $MnCl_2 \cdot 4H_2O$, 0.76 µM $ZnSO_4 \cdot 7H_2O$, 0.2 µM $CuSO_4 \cdot 5H_2O$, 0.28 µM $Na_2MoO_4 \cdot 2H_2O$, 51.7 µM Fe-EDDHA). To assess the effect of Fe supply, the same nutrient solution was used but with a lower or higher Fe concentration (0.1 µM or 1 mM Fe-EDDHA). After 5 days of Fe treatment, plants were infected with *M. oryzae* spores (see below for inoculation method).

Perls Staining and DAB/H_2O_2 Intensification

Rice leaves (mock- and blast-inoculated, 48 hours post-infection [hpi]) were stained with Prussian blue dye according to (Roschzttardtz *et al.* 2009) with some modifications. Briefly, rice leaves were vacuum-

infiltrated in a fixing solution (chloroform:methanol:glacial acetic acid; 6:3:1, v/v) for 1 h and incubated overnight at room temperature. After washing with distilled water (three times), samples were vacuum-infiltrated with a pre-warmed (37ºC) staining solution (4% HCl and 4% K-ferrocyanide at equal volumes) for 1 h, incubated 1 hour more at 37ºC in the same solution without vaccuum and washed three times with distilled water (Perls staining). For DAB intensification reaction, samples were incubated in a methanol solution (0.01 M NaN, 0.3% [v/v] H_2O) for 1 h, washed with 0.1 M phosphate buffer pH 7.2, then incubated with the intensification solution (0.025% [w/v] DAB [Sigma], 0.005% [v/v] H_2O in 0.1 M phosphate buffer, pH 7.2) for 15 min. The reaction was stopped by washing with distilled water. Leaves were mounted in glycerol 50% in glass slides and observed under a microscope (AixoPhot DP70 under with light).

Chlorophyll Content

The mean of 10 readings from the chlorophyll meter (SPAD 502 Plus Chlorophyll Meter, Spectrum Technologies) was obtained from the third leaf of rice plants grown in different Fe concentrations. The measurement was taken at the same position in all leaves.

Blast Resistance Assays

The fungus *M. oryzae* (strain Guy-11, courtesy of Ane Sema) was grown in Complete Media Agar (CMA, 9 cm plates, containing 30 mg/L chloramphenicol) for 15 days at 28ºC under a 16 h/8 h light/dark photoperiod condition. *M. oryzae* spores were prepared as previously described (Campo *et al.* 2013). Soil-grown plants (3-4 leaf stage) were infected by two different methods, 1) whole-plant spray inoculation assays (Sesma and Osbourn 2004), and 2) drop inoculation on detached leaves (Coca *et al.* 2004). Briefly, the spray inoculation method consisted

of spraying whole rice plants with a *M. oryzae* spore suspension (10^5 spores/ml; 0.2 ml/plant) by using an aerograph at 2 atmospheres of pressure. Plants were maintained overnight in the dark under high humidity. For the drop inoculation method, the second detached leaf was placed into square plate dishes (12 leaves/plate) with 1% (w/v) water agar containing kinetin (2 mg/l). Then, Whatman filter paper discs saturated with a *M. oryzae* spore suspension (10^4-10^6 spores/ml) were placed onto the upper face of the leaf for 60 h. The percentage of leaf area affected by blast lesions was determined at 4 days (drop-inoculated leaves) or 7 days (spray-inoculated leaves) post-inoculation with *M. oryzae* spores by using the APS Assess 2.0 program (Lamari 2008).

Expression Analysis

Total RNA was extracted from plant tissues by using TRizol reagent (Invitrogen). For northern blot analysis of rice miRNAs, RNAs were fractionated in a 17.5% denaturing polyacrylamide gel containing 8M urea, transferred to nylon membranes and probed with a γ^{32}P-ATP end-labeled miR7695.3-3p oligonucleotide (**Additional file 2: Table S1**). Blots were pre-hybridized and hybridized in Perfect-Hyb Plus buffer (Sigma) at 42ºC. Hybridization signals were detected by using STORM Phosphorimager (GE Healthcare).

For quantitative RT-PCR (RT-qPCR), the first complementary DNA was synthesized from DNase-treated total RNA (1 µg) with High Capacity cDNA Reverse Transcription (Life technology, Applied Biosystems). Amplification involved 2 µl cDNA (5 ng/µl) in optical 96-well plates (Roche Light Cycler 480; Roche Diagnostics, Mannheim, Germany) with SYBR Green I dye and gene-specific primers (**Additional file 2: Table S1**). The *Ubiquitin1* gene (Os06g0681400) was used to normalize transcript levels.

5'-RLM-RACE

5′ RNA ligase-mediated rapid amplification of cDNA ends (5' -RLM-RACE) was done using a GeneRacer^{TM} kit according to the manufacturer's instructions (Invitrogen, CA) but omitting the dephosphorylation and decapping steps. Briefly, 3 µg of DNAse-treated total RNA was ligated to a GeneRacer Oligo RNA Adapter. First-strand cDNA was synthesized using oligo-dT. Specific primers were used to amplify 5' ends by nested PCR from cDNA (**Additional file2: Table S1**). The nested PCR products were separated on a 2% agarose gel, gel purified, ligated to a Zero Blunt TOPO vector (Invitrogen, CA) transformed into Topo 10 cells and sequenced to determine the cleavage site in target genes. Specific control were done using the specific primers.

RNA-seq Library Sample Preparation and Sequencing

Total RNA was extracted from rice leaves that had been treated or not with a *M. oryzae* spore solution following the whole-plant infection method (10^5 spores/ml, 0.2 ml/plant, at 48 hpi) with the Maxwell 16 LEV Plant RNA Kit (Promega). Raw reads were checked for quality by using FastQC v0.11.3 (www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to check quality of raw reads; adapters were trimmed and removed with Trimmomatic v0.33 (Bolger *et al.* 2014) (minimum quality score 35, minimum length 25). Reads obtained were mapped to the reference rice genome (MSU 7.0) provided with the reference gene annotation file (RGSP 7.0) by using STAR (v2.4.0j) (Dobin *et al.* 2013). Reads with mapping quality (MAPQ) <30 were removed. FeatureCounts (v1.4.5-p1) (Liao *et al.* 2014) was used to perform read summarization at the gene level, with the strand-specific option "reversely stranded". Statistical analysis of read counts was performed with R, with the HTSFilter package (Rau *et al.* 2013) to remove low-expressed genes and the edge R package (Robinson *et al.* 2009) for differential expression analysis. To identify genes with significant difference in expression, a

FDR cutoff < 0.05 and log2FC 1≤ or ≥ 1 was applied. Gene Ontology (GO) enrichment of differentially expressed genes involved Singular Enrichement analysis (SEA) using the AgriGO webtool ($p<0.01$ Fisher's test, TIGR genemodel) (http://bioinfo.cau.edu.cn/agriGO/) (Du *et al.* 2010). Enriched GO terms were grouped, summarized and 2D-plotted by semantic clustering with the online analysis tool ReviGO (http://revigo.irb.hr/) (Supek *et al.* 2011).

Quantification of Rice Diterpene Phytoalexins

Leaf segments were collected from mock and *M. oryzae*-infected plants. Three biological replicates with two technical replicates each were performed. Approximately 200-300 mg of fresh plant material was soaked in 40 vol of 70% methanol and incubated at 4ºC overnight with constant rotation. A 1ml aliquot was centrifuged at maximum speed to remove cell debris. Phytoalexins were quantified using 5µl of the extract by LC-MS/MS as described (Miyamoto *et al.* 2016). Significant differences in phytoalexin accumulation were evaluated with ANOVA.

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SUPPLEMENTAL FIGURES

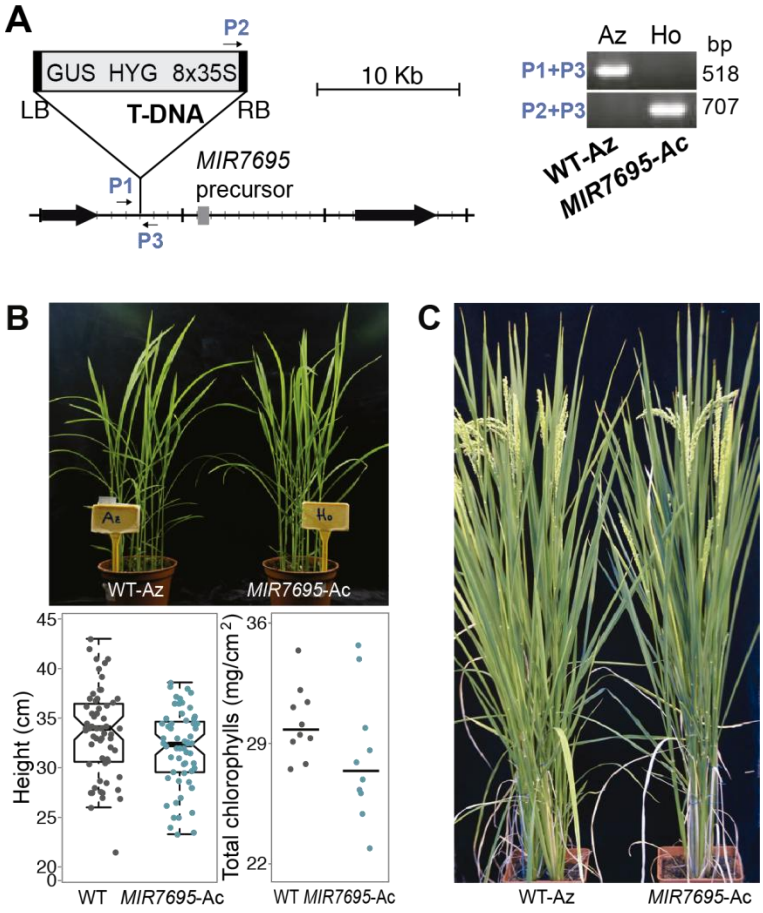

Figure S1. Characterization and Phenotype of *MIR7695*-Ac and wild-type azygous (WT-Az) plants. **(A)** Schematic representation of the T-DNA insertion mutant from the TRIM collection (M0107013). GUS, β-glucuronidase; HYG, hygromycin phosphotransferase; 8x35S, Cauliflower mosaic virus 35S enhancer; RB, Right border; LB, Left border. (left panel) PCR genotyping of mutant plants with specific primers (P1, P2, P3; Additional file 2: Table S1) (right panel). Ho, homozygous for the T-DNA insertion; Az, segregated azygous. **(B)** Soil-grown *MIR7695*-Ac and WT-Az plants were grown under greenhouse conditions for 3 weeks(s) and under a 14 h/10 h light/dark cycle, at 26 ± 2ºC. Height and chlorophyll content of *MIR7695*-Ac and WT-Az plants (left and right panels, respectively). Box plots show median, quartiles (boxes) and range (whiskers) for plant height measurements (n = 60). Notches indicate the 95% confidence interval of the median. Differences between *MIR7695*-Ac and WT-Az plants were not statistically significant. **(C)** *MIR7695*-Ac and WT-Az plants at the maturity stage. Plants were grown for 3 months as in (a).

Figure S2. Resistance of *MIR7695*-Ac mutant plants to *M. oryzae* infection. Disease resistance was determined by local inoculation of detached leaves with *M. oryzae* spore suspension at 10^5 spores/ml. Leaves were photographed at 4 days post-inoculation. Panel below, percentage of leaf area affected by blast lesions as determined by image analysis (APS Assess 2.0) (Lamari 2008). Data are mean ±SE (n=7). Three independent infection assays were performed with similar results. *P<0.05.

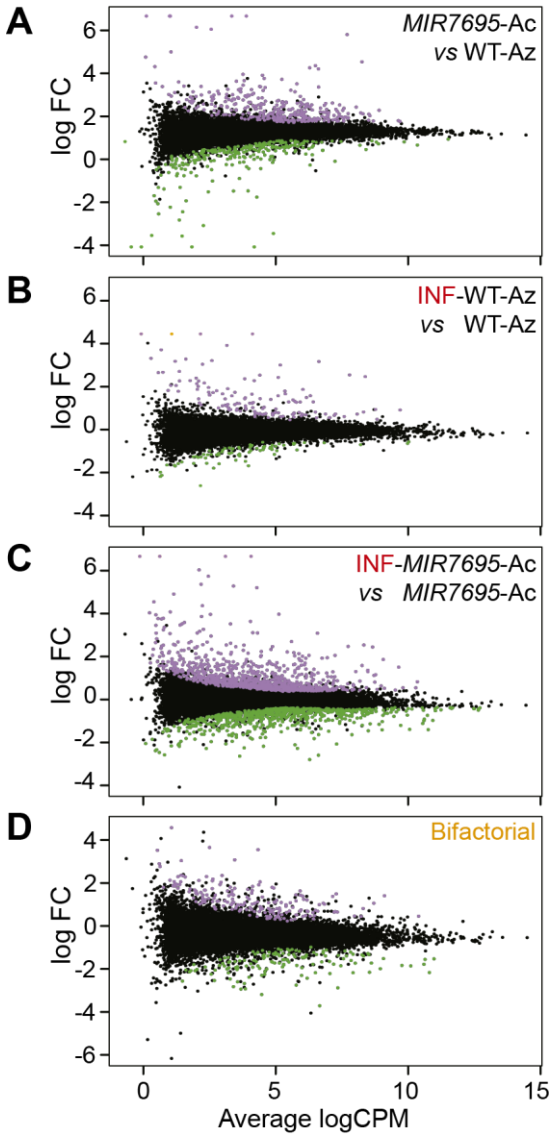

Figure S3. Differentially expressed genes (DEGs) in leaves of *MIR7695*-Ac mutant plants relative to WT-Az plants, under non-infection or infection. Leaves of 3-week-old rice plants were mock-inoculated (WT-Az, *MIR7695*-Ac) or inoculated with *M. oryzae* spores (INF-WT-Az, INF-*MIR7695*-Ac). Leaves were collected at 48 h post-inoculation. MA Plots of logarithmic fold changes (LogFCs) to average count size in RNA-seq analysis of *MIR7695*-Ac plants vs WT-Az plants across different conditions. DEGs are highlighted in purple (upregulated) and green (downregulated) ($p<0.05$, FDR<0.05, n=2).

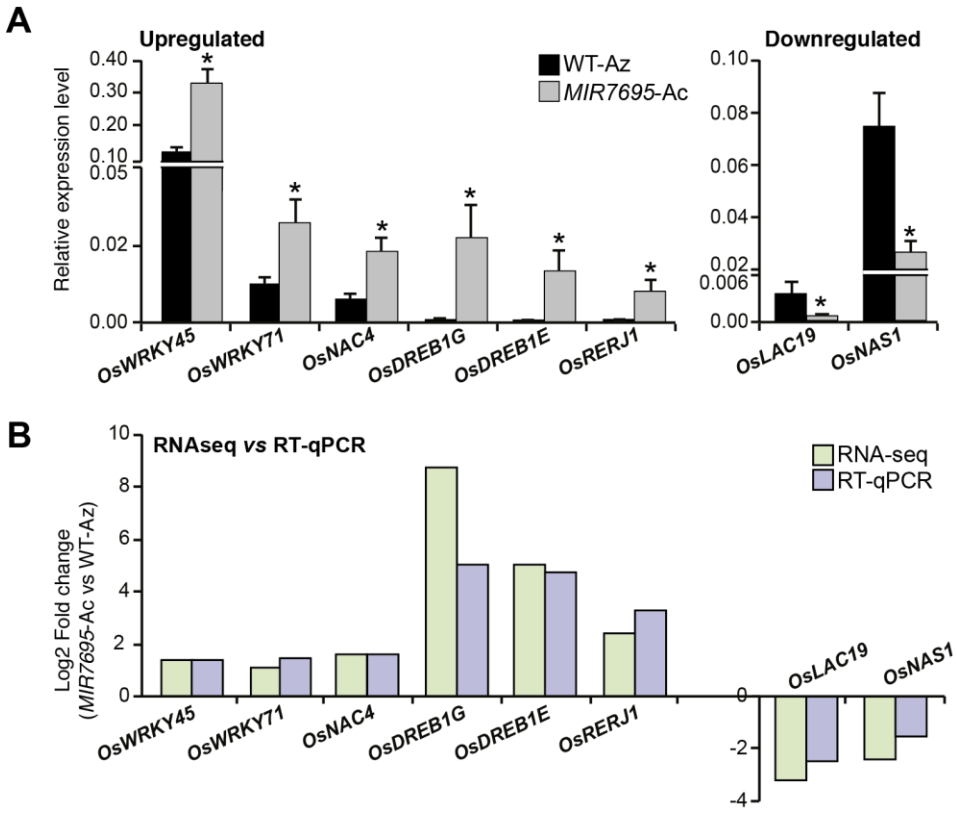

Figure S4. Validation of RNAseq data by qRT-PCR. Leaves of 3-week-old WT-Az and *MIR7695-Ac* plants were used for RT-qPCR analysis (n=3). **(A)** Expression of selected genes upregulated (left panel) or downregulated (right panel) in *MIR7695*-Ac vs WT-Az by RNA-seq. Data are mean ± SE (n=3) and were normalized to the rice *Ubiquitin* (Os06g0681400). Gene-specific primers are in Additional file 2: Table S1 *P **(B)** Comparison of RNA-seq and RT-qPCR fold change values obtained for DEGs in (a).

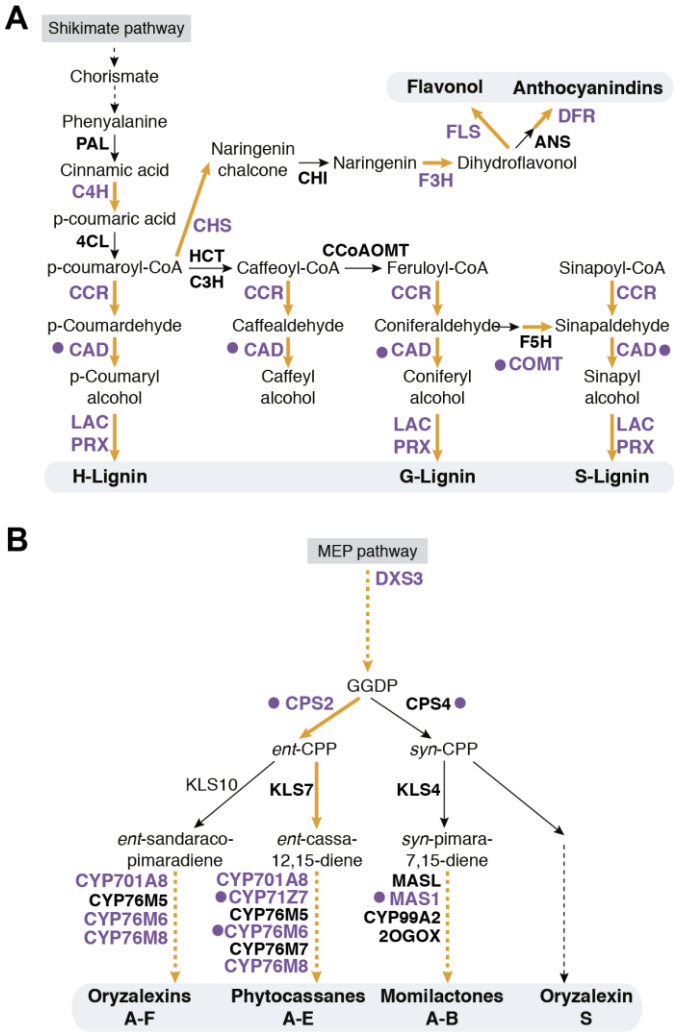

Figure S5. Pathways for the biosynthesis of phenylpropanoids (a) and diterpenoid phytoalexins (b) in rice. RNA-seq analysis of genes upregulated in *MIR7695*-Ac versus WT-Az plants during *M. oryzae* infection (in purple; log2FC ≥ 1; pval <0.05; FDR <0.05). **(A)** Simplified scheme of the monolignol and flavonoid branches of the phenylpropanoid metabolism pathway. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3 hydroxylase; FLS, flavonol synthese; ANS, Anthocyanidin synthese; DFR, dihydroflavonol reductase; HCT, hydroxycinnamoyl transferase; C3H, coumarate 3-hydroxylase; CCoAOMT, caffeoyl/CoA-3-O metheltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid 3-Omethyltransferase; LAC, laccase; PRX, peroxidase. RT-qPCR of upregulated genes (purple dot) (see Fig. 7). **(B)** Diterpenoid phytoalexin biosysnthesis pathway. CPS, ent-Copalyl diphosphate synthase; KSL, kaurene synthase-like; MAS, momilactone A synthase, CYP, Cytochrome P450. RT-qPCR of upregulated genes (purple dot) (see Fig. 8).

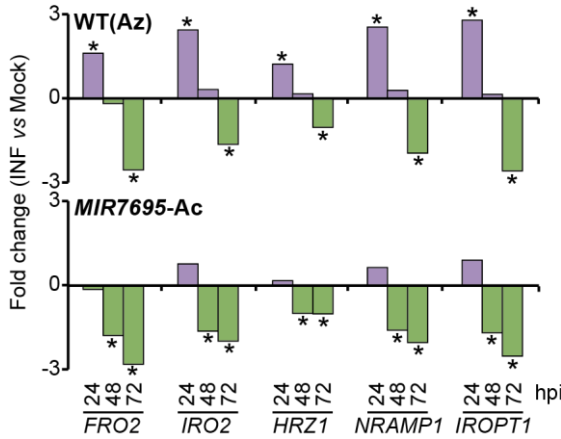

Figure S6. RT-qPCR analysis of expression pattern of Fe homeostasis genes (*OsFRO2*, *OsIRO2*, *OsHRZ1*, *OsNRAMP1*, *OsIROPT1*) in WT-Az and *MIR7695*-Ac plants with M. oryzae infection. The plant material was treated as in Fig. 7. Data are mean±SE (n=3) after normalization to Ubiquitin expression. *P<0.05.

Chapter II

Iron treatment induces defense responses and disease resistance against *Magnaporthe oryzae* in rice

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ABSTRACT

Background: Iron is an essential micronutrient required for plant growth and development. The impact of iron in plant-pathogen interactions is also well recognized. However, the molecular basis underlying the effect of plant iron status and immune function in plants is poorly understood. Here, we investigated the impact of treatment with high iron in rice immunity at the cellular and molecular level.

Results: We show that treatment with high iron confers resistance to infection by the blast fungus *M. oryzae* in rice. Histochemical staining of *M. oryzae*-infected leaves revealed that iron and Reactive Oxygen Species (ROS) accumulate at high levels in cells in the vicinity of the infection site. During pathogen infection, a stronger induction of defense-related genes occurs in leaves of iron-treated plants. Notably, a superinduction of phytoalexin biosynthetic genes, both diterpene phytoalexins and sakuranetin, is observed in iron-treated plants during pathogen infection. As a consequence, phytoalexin accumulation was higher in iron-treated plants compared with control plants. Transcriptional alterations of iron homeostasis-related genes and a reduction in apoplastic iron content were observed in leaves of Fe-treated rice plants.

Conclusions: These results illustrate that the iron status plays a key role in the response of rice plants to pathogen infection, while reinforcing the notion that iron signaling and defense signaling must operate in a coordinated manner in controlling disease resistance in plants. This information provides a basis to better understand the molecular mechanisms involved in rice immunity.

INTRODUCTION

Iron (Fe) is an essential element required for a wide range of biological functions during plant growth and development. Plants require Fe for photosynthesis, mitochondrial respiration and hormone biosynthesis, among other processes. Fe is associated to cellular reduction-oxidation reactions (redox reactions) and electron transfer chains. It is also a co-factor for a variety of proteins mediating redox reactions. Fe deficiency results in chlorosis, poor growth and reduced yields (Hänsch *et al.* 2009). Although Fe is a constituent of approximately 5% of Earth's crust, the availability of Fe to plants is usually very low (Connorton *et al.* 2017). Therefore Fe deficiency is a common problem in agricultural systems worldwide. Fe bioavailability is limited in aerobic conditions and high pH soil conditions where Fe, is predominantly found in the form of insoluble ferric form (Fe^{3+}), forming hydroxide complexes poorly available for living organisms. In contrast, under reductive conditions, Fe is present as the soluble ferrous form (Fe^{2+}) (Connorton *et al.* 2017).

Rice has evolved two distinct strategies to take up Fe from the soil, referred to as Strategy I and Strategy II (Bandyopadhyay and Prasad 2021). Dicotyledonous and non-graminaceous monocotyledonous species use **Strategy I**, or **reduction strategy** to increase Fe^{3+} solubility. It involves the secretion of phenolics and soil acidification by the release of protons into the rhizosphere, and is mediated by Phenolics Efflux Zero 1 (PEZ1) and H^+-ATPase, respectively. Iron is subsequently reduced from the ferric (Fe^{3+}) form to a more soluble ferrous form (Fe^{2+}) by a ferric reductase-oxidase (FRO) and then it is transported across the plasma membrane into the root cells by Iron-Regulated Transporter (IRT1, IRT2). The **Strategy II**, also called the **chelation strategy**, is used by graminaceous species. Here, roots secrete Fe^{3+} chelators of the mugineic acid (MA) family called phytosiderophores (PS). This pathway implies the biosynthesis of the non-proteinaceaous amino-acid nicotianamine (NA) from S-adenosyl-L-methionine by NA synthase (NAS) and then NA

is synthesized into 2'-deoxymugineic acid (DMA), the precursor of MAs, by NA aminotransferase (NAAT), and DMA synthase (DMAS). Secretion of MAs from rice roots to the rhizosphere is mediated by *OsTOM1* (Nozoye *et al.* 2011).

Rice is typically cultivated under anaerobic conditions (paddy fields) where abundant Fe^{2+} is readily available to the plant, especially in soils with a low pH in which Fe^{3+} is reduced to the more soluble ferrous ion Fe^{2+}. Under such conditions, unlike other graminaceous plants, the absorption of Fe^{2+} by the rice roots might cause severe Fe toxicity. Thus, excessive accumulation of Fe^{2+} can be harmful to the plant (Connorton *et al.* 2017; Schmidt *et al.* 2020). The formation of deleterious reactive oxygen species (ROS) through redox reactions between ferric (Fe^{3+}) and ferrous (Fe^{2+}) (Fenton reaction) leads to the oxidation of biomolecules (lipids, proteins, DNA) and damage to cellular structures, and eventually, cell death (Turhadi *et al.* 2019). Therefore, Fe acquisition, use and storage must be tightly regulated at the cell and tissue level to provide enough amounts for the plant metabolism while preventing accumulations of deleterious ferrous Fe. Organelles are crucial compartments for Fe storage and sequestration within the plant cell. In particular, vacuoles play a major role in accumulating Fe excess, and releasing Fe into the cytosol, if required for metabolism (Kar and Panda 2020). Also, plant ferritins serve to store Fe and for protection against Fe-catalyzed ROS production (Arosio *et al.* 2009). The storage capacity of plant ferritins (e.g *Atfer1-4*, *Osfer1-2*) is of aprox. 4500 Fe atoms in the bioavailable and non-toxic form of Fe (Briat *et al.* 2010). Plants regulate Fe concentration in different subcellular compartments in a homeostatic way through dynamic processes to avoid Fe toxicity (Müller *et al.* 2015).

Because Fe participates in the generation of ROS, and ROS production is generally associated with resistance to pathogen infection, it is not surprising that Fe availability affects the outcome of plant pathogen interactions (Aznar *et al.* 2015; Dangol *et al.* 2019; Herlihy *et al.* 2020; Liu *et al.* 2007; Torres *et al.* 2005; Ye *et al.* 2014). As an example, resistance to the necrotrophic pathogens *Dickeya dadantii* and *Botrytis*

cinerea was observed in Fe-starved *Arabidopsis* plants supporting that crosstalk between Fe deficiency response and immunity occurs in Arabidopsis (Kieu *et al.* 2012). Fe-starved maize plants were found to be unable to produce ROS in response to *Colletotrichum* infection which correlated with increased susceptibility to this fungal pathogen (Ye *et al.* 2014). However, our understanding on the regulatory mechanisms that mediate plant Fe homeostasis and plant immunity is still limited.

During pathogen infection, there is a competition between the host and the pathogen for Fe as the pathogen must acquire this vital element from host tissues, while the plant might interfere with Fe acquisition as a defense strategy during infection. On the one side, plant pathogens employ diverse strategies for Fe acquisition from the host plant (i.e. secretion of high-affinity Fe-binding siderophores) (Aznar *et al.* 2015). On the other side, plants have evolved mechanisms to sequester iron from pathogens during infection, a phenomenum that was originally described in animals, the so called "nutritional immunity" (Weinberg 1975). The host plant might also capitalize the toxicity of iron through local accumulation of Fe leading to activation of an oxidative burst that can be toxic for the invading pathogen (Aznar *et al.* 2015).

Rice ranks among the 10 most important crops worldwide, with greater importance for developing countries in terms of food security and alleviation of malnutrition. The rice blast disease caused by the ascomycete fungus *Magnaporthe oryzae* is the most devastating fungal disease of cultivated rice worldwide (Dean *et al.* 2012; Fernandez and Orth 2018; Wilson and Talbot 2009). *M. oryzae* is an ascomycete fungus with a hemibiotrophic lifestyle that involves initial proliferation inside living host cells before switching to a destructive necrotrophic mode (Fernandez and Orth 2018; Wilson and Talbot 2009). Many studies have been carried out during the last years to elucidate the molecular and cellular mechanisms implicated in the rice response to *M. oryzae* infection (Fernandez and Orth 2018). In previous studies, we reported that Fe supply positively impacts blast disease resistance in hydroponically-

grown rice plants (Peris-peris *et al.* 2017) supporting crosstalk between iron signaling and immune signaling in rice.

In this work, we investigated the effect of treatment with high Fe on resistance to infection by *M. oryzae* in rice at the molecular and cellular level. Histological staining of *M. oryzae*-infected leaves revealed ROS and Fe accumulation in cells which are located in the vicinity of the infection sites. Treatment with high Fe increased resistance to *M. oryzae* which was accompanied by stronger induction of defense gene expression. Fe-treated rice plants also showed a higher induction of phytoalexin biosynthesis genes during pathogen infection, which correlated well with accumulation of major rice phytoalexins in the infected leaves, both diterpenoid phytoalexins and sakuranetin. *M. oryzae* infection also causes transcriptional regulation of genes involved in Fe homeostasis in rice leaves which was accompanied by alterations in total Fe and apoplastic Fe content. Collectively, these results further support links between the Fe status in the rice plant and resistance to infection by the blast fungus *M. oryzae*.

RESULTS

Treatment with high Fe enhances resistance to *M. oryzae* infection in rice plants.

Resistance to infection by *M. oryzae* was investigated in soil-grown rice plants that have been treated with high Fe. For this, the rice plants were grown under sufficient Fe supply (50 µM Fe) for 16 days. Then, half of the plants continued growth under sufficient Fe while the other plants were supplied with 1 mM Fe (henceforth control and high-Fe plants, respectively). Two different periods of treatment with high-Fe were used, 5 and 19 days. After 5 days of treatment, no phenotypic differences were observed between control and high-Fe plants (**Supplemental Figure S1A**). Root and leaf biomass (fresh weight), as well as chlorophyll content did not differ between control and high-Fe plants (**Supplemental Figure S1B**). To note, at this time of treatment, the Fe level in tissues of high-Fe were higher than that in control plants (root, stem and leaves) (**Supplemental Figure S1C**). High Fe treatment caused a significant down-regulation in the expression of typical iron deficiency-inducible genes (*OsIRO2, OsNAS1, OsNAS2*) in the rice roots, thus, supporting that the plant perceives and respond to Fe treatment (**Supplemental Figure S1D)**. When Fe treatment was carried out for a longer period of time (e.g. 19 days of treatment with 1 mM Fe), the plants developed symptoms of Fe toxicity in leaves (**Supplemental Figure S2A**). At 19 days of treatment, the high-Fe plants also showed a significant reduction in root and leaf biomass (fresh weight) and chlorophyll content compared control plants (**Supplemental Figure S2B**). To avoid that toxic effects caused by Fe accumulation could confound our results on disease resistance, a period of 5 days of treatment with high Fe (1mM Fe) was chosen in this work. Under these experimental conditions, no penalty on plant growth was observed.

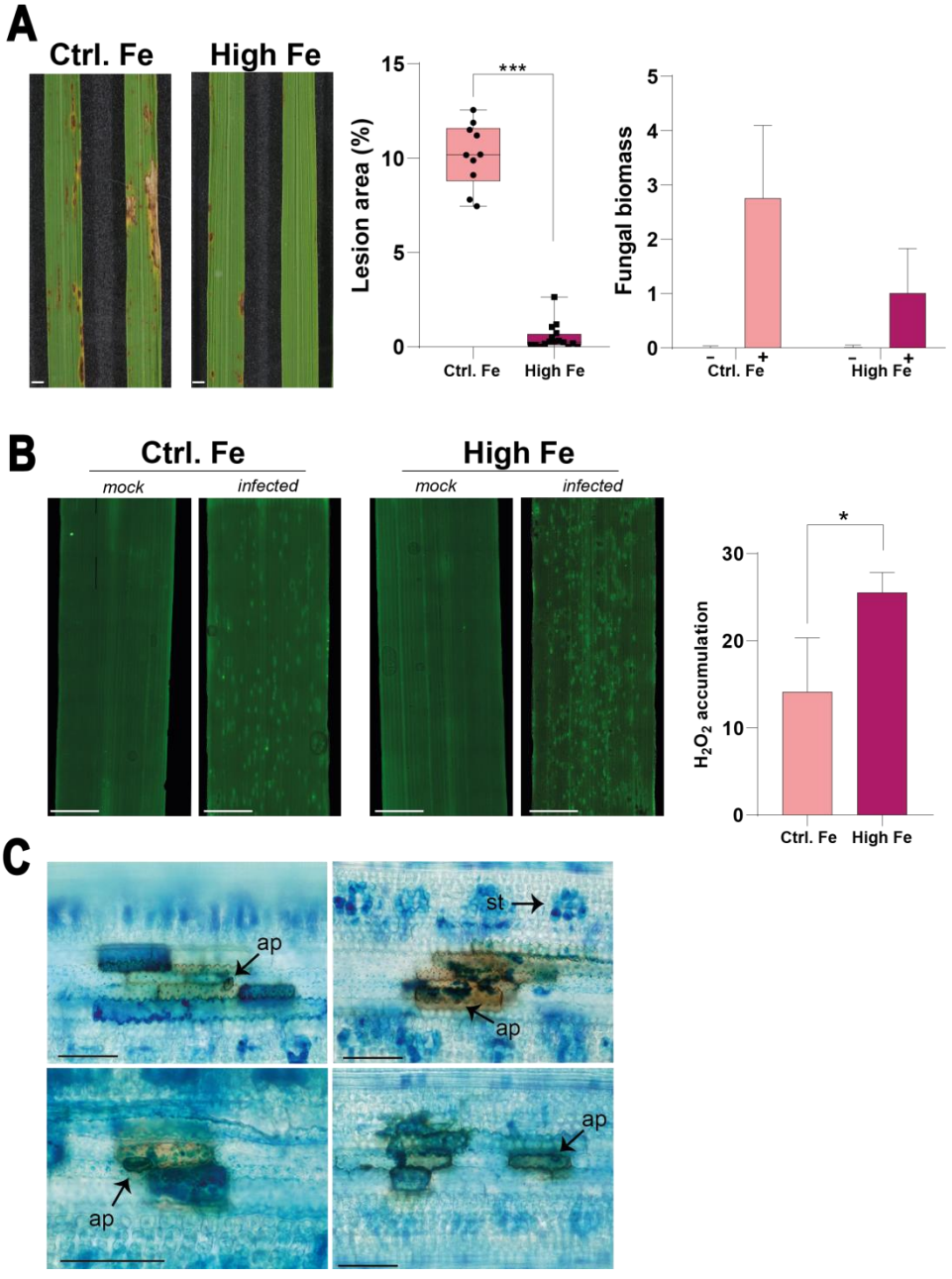
Blast resistance was assayed on high-Fe (after 5 days of treatment) and control Fe plants. Compared with control plants, high-Fe plants

consistently exhibited higher resistance to *M. oryzae* infection as revealed by visual inspection of disease symptoms, quantification of diseased leaf area and measurement of fungal biomass in pathogen-infected leaves (**Figure 1A**). These results were substantially similar to those previous reported in hydroponically-grown rice plants (Peris-peris *et al.* 2017). Thus, treatment with high Fe enhances resistance to *M. oryzae* infection in rice plants.

A generalized plant defense against pathogen attack is the production of reactive oxygen species (ROS) (Torres 2010). Among ROS, H_2O_2 accumulation provokes localized cell death around the site of infection to limit the spread of the pathogen. Indeed, H_2O_2 is considered an important molecule in regulating plant immune responses (Torres *et al.* 2006). On this basis, we investigated whether treatment of rice plants with high Fe has an effect on ROS accumulation potentially contributing to the observed phenotype of blast resistance. Histochemical detection of ROS, mainly H_2O_2, was carried out using the fluorescent probe H_2DCFDA (2', 7' dichlorofluorescein diacetate) (Fichman *et al.* 2019) in leaves of control and high-Fe plants inoculated, or not, with *M. oryzae* spores. In the absence of pathogen infection, ROS accumulation was barely detected in control and high-Fe plants (**Figure 1B**, mock). Upon pathogen challenge, discrete regions accumulating ROS (H_2O_2) could be observed in control and high-Fe plants, but the DCFDA-fluorescent signals were more intense and abundant in high-Fe plants relative to control plants (**Figure 1B,** infected). The regions accumulating ROS, most probably, correspond to the *M. oryzae* infection sites. Quantification of H_2DCFDA fluorescence using ImageJ software confirmed higher accumulation of ROS in *M. oryzae*-infected leaves of high-Fe plants than in *M. oryzae*-infected leaves of control plants (**Figure 1B,** right panel**).**

Knowing that rice plants grown under high Fe supply showed ROS accumulation during *M. oryzae* infection, and that Fe catalyzes the Fenton reaction to produce ROS (Krohling *et al.* 2016), it was of interest to investigate the sites of Fe accumulation in *M. oryzae*-infected leaves in Fe-treated rice plants. For this, we performed double staining using DAB

(3, 3'-diaminobenzidine) for ROS detection followed by Perls staining for Fe detection. The Perls reagent (potassium ferrozianide) has been widely used for histochemical detection of Fe^{3+} ions in plant tissues, based on the capability of Fe^{3+} to react with potassium ferrozianide to produce Prussian blue (Roschzttardtz *et al.* 2010, 2013). For this study, we focused on high-Fe plants, these plants showing resistance to *M. oryzae* infection. Double staining of *M. oryzae*-infected leaves of high-Fe plants revealed ROS (brown) accumulation at the invaded cells, whereas Fe^{3+} (blue) accumulated in the invaded cells as well as in contiguous cells (**Figure 1C**). In previous studies, Dangol *et al.* (2019) described that Fe^{3+} and ROS (e.g. H_2O_2) accumulate in rice leaves during infection with an avirulent strain of *M. oryzae*. In non-infected rice leaves, iron accumulates at stomata (**Supplemental Figure S3**; similar results were previously reported by Sánchez-Sanuy *et al.* 2019). Resistance to *M. oryzae* infection in Fe-treated rice plants can then be explained by a localized accumulation of Fe and H_2O_2 at the sites of pathogen penetration.


A
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High Fe
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15
10
5
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Ctrl. Fe
High Fe
Fungal biomass
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3
2
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mock
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mock
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30
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Figure 1. Resistance to *M. oryzae* infection in rice plants that have been grown under high Fe supply. Rice plants were grown in soil for 16 days under control Fe supply (0.05 mM Fe) and then supplied with high Fe (1 mM Fe; control plants) for 5 days more. Control plants were allowed to continue growth under control Fe supply. Rice plants were inoculated with a conidial suspension of *M. oryzae* spores (5 x 10^5 spores/ml), or mock-inoculated. Data from one representative experiment of four independent experiments are presented. (**A)** Disease symptoms at 7 days post-inoculation (dpi). Right panels, percentage of diseased area at 7 dpi (n=10). Fungal biomass was quantification by qPCR using specific primers of the *M. oryzae 28S* ribosomal gene (relative to the rice *Ubiquitin 1* gene Os06g46770) at 7 dpi, (mock (-) and *M. oryzae* (+). Data are mean ± SEM (n=10). Asterisks indicate statistical significant differences calculated by *t*-test (*** indicate $p < 0.001$). (**B)** ROS accumulation in control and high-Fe rice plants under non-infection and infection conditions. ROS (H_2O_2) was detected using the fluorescent probe H_2DCFDA at 48 hpi in mock-inoculated (mock) and *M. oryzae*-inoculated (infected) plants. Bars correspond to 2 mm. Right panel, Image J software was used for quantification of ROS fluorescence of three independent experiments (100 fields, each). Asterisks indicate statistical significant differences calculated by two-way ANOVA (*$p < 0.05$) (**C)** Accumulation of ROS (in brown) and iron (Ferric ions, Fe^{3+}, in blue) in *M. oryzae*-inoculated leaves of high-Fe plants. The third leaf was stained with DAB (for ROS staining) followed by Prussian Blue (Perls reagent, for Fe staining) at 24-48 hpi. Bars correspond to 50 μm. Ap: apressorium; St, stomata.

High Fe supply alters the transcriptome of rice plants

Most research to date on the rice response to Fe supply focused on transcriptional regulation of gene expression in roots (Ogo *et al.* 2014). Relatively less is known about transcriptional alterations induced by Fe treatment on rice leaves. Even less is known about the impact of Fe treatment on immune responses of rice plants to pathogen infection. Accordingly, in this work we initially performed a comparative transcriptome analysis of leaves from control and high-Fe plants. Differentially expressed genes (DEGs) were identified based on significance level (FDR ≤ 0.05) and $\log_2$ fold change (FC) with a threshold of FC $\geq + 0.5$ and FC $\leq - 0.5$ for up-regulated and down-regulated genes, respectively. Using these criteria, only 48 genes were found to be up-regulated in high-Fe plants compared to control plants, while 191 genes were down-regulated (the full list of DEGs is presented in **Supplemental Table S1a**). To note, genes whose expression is typically induced by Fe deficiency in rice roots where found to be repressed in leaves of high-Fe plants relative to control plants (**Figure**

2A, Supplemental Table S1b). They included: *OsIMA1* and *OsIMA2* (*iron deficiency-inducible peptide- IRON MAN*), *OsIRO2* and *OsIRO3* (*iron-related transcription factor 2 and 3*), *OsHRZ1* and *OsHRZ2* (Iron-binding Haemerythrin RING ubiquitin ligase). Other genes involved in iron homeostasis that are down-regulated in leaves of high-Fe rice plants are those encoding the plasma membrane iron transporters *OsOPT7* (*iron-deficiency-regulated oligopeptide transporter 7*), *OsNRAMP1* (*Natural Resistance-Associated Macrophage Protein 1*) and *OsVMT* (a vacuolar mugineic acid transporter) (**Figure 2A**). *OsFER2* (*FERRITIN 2*) expression was induced in leaves of high-Fe plants (**Figure 2A**). Ferritins are the primary Fe-storage proteins (in the form of Fe^{3+}) and contribute to protection of plants against *Fe*-induced oxidative stress.

Treatment with high Fe was also accompanied by alterations in the expression of genes implicated in detoxification and protection against oxidative stress. An important number of peroxidases were found to be misregulated by Fe treatment, which were either up-regulated (*OsPOX22.3, Prx62*) or down-regulated (*Prx11, Prx15, Prx16, Prx38, Prx81, Prx83, Prx89*) (**Figure 2A**). *OsPOX22.3* was found to accumulate in incompatible reactions of rice plants to *M. oryzae* (Faivre-Rampant *et al.* 2008). Besides catalyzing the decomposition of hydrogen peroxides**,** peroxidase enzymes have the capacity to produce H_2O_2, a process that is considered to be the source of H_2O_2 required for cell wall lignification. A Metallothionein-like protein (*OsMT3a*) and a glycolipid transfer protein involved in ceramide transport (*OsGLTP*) were induced also up-regulated in high-Fe plant compared with control plants (**Figure 2A**). Metallothioneins are metal-binding proteins implicated in metal detoxification and scavenging of ROS (Hassinen *et al.* 2011), while ceramides have been proposed to play a role in the induction of cell-death in rice, also linked to ROS accumulation (Zhang *et al.* 2020).

We also noticed that genes related to stress responses were misregulated genes in Fe-treated plants in the absence of pathogen infection, including genes for which a role in blast resistance has been described (**Figure 2A**). Among them, there were distinct members of

several families of *Pathogenesis-Related* (*PR*) genes. Whereas *OsPR1b* was up-regulated in high-Fe plants compared with control plants, other *PR* genes were found to be down-regulated in these plants, such as *OsCht11* (PR3 family of PR proteins), *OsChib3a* (PR8 family), *JIOsPR10* (*Jasmonate Inducible PR10*; PR10 family) and four *OsGLP* genes (Germin-Like Proteins; PR14 family) (**Figure 2A**).

It is well known that the WRKY45 transcription factor plays a crucial role in resistance to *M. oryzae* infection, and that *WRKY45* overexpression confers resistance to *M. oryzae* (Shimono *et al.* 2012). Surprisingly, *OsWRKY45* was found to be down-regulated in high-Fe plants compared with control plants. Down-regulation of *PR* and *OsWRKY45* was in apparent contradiction with the phenotype of blast resistance that is observed in rice plants grown under high Fe conditions. Other defense-related genes down-regulated by Fe treatment were *OsBBI*, *OsJAC1* which are implicated in resistance to pathogen infection (Li *et al.* 2011; Weidenbach *et al.* 2016). Treatment with Fe also caused a general repression of key genes involved in plant cell wall biosynthesis (**Figure 2A**). Finally, RT-qPCR analysis confirmed RNA-Seq data (**Figure 2B**).

Together, these findings indicated that treatment with Fe was accompanied by alterations in the expression of genes that are typically activated by *M. oryzae* infection, even though the rice plants were not challenged with *M. oryzae*. Intriguingly, many of these genes showed down-regulated in high-Fe plants compared with control plants, although high-Fe plants exhibited enhanced resistance to *M. oryzae* infection. We then reasoned that blast resistance in high-Fe plants might rely more on the stronger induction of defense responses upon pathogen infection rather than constitutive defenses.

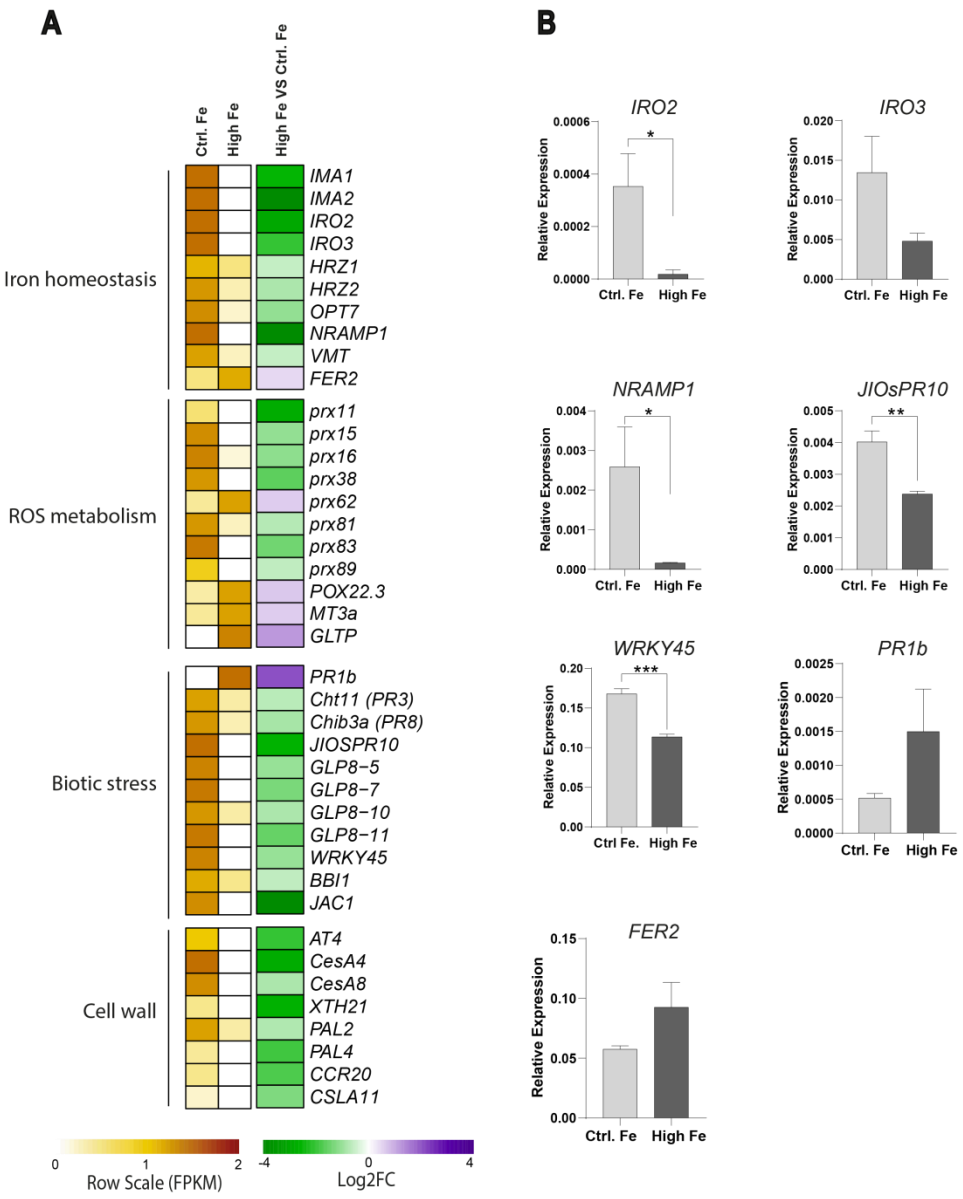


Figure 2. Differentially expressed genes in leaves of high-Fe plants relative to control plants. **(A).** Heatmaps showing differentially expressed genes (DEGs) by RNASeq analysis. Right panel, expression level (row scaled FPKM) is represented from pale yellow (less expressed) to brown (more expressed). Left panel, up-regulated genes (Log_2 fold change (FC) $\geq$ + 0.5; purple) and down-regulated genes (Log_2 FC $\geq$ - 0.5; green) DEGs. Data represented correspond to the mean of two biological replicates, each biological replicate consisting in a pool of 5 leaves from individual plants. The full gene name and ID of DEGs are indicated in **Supplemental Table S1a, S1b**. **(B)** Expression of differentially expressed genes identified by RNASeq analysis. Transcript levels were determined by RT-qPCR analysis in leaves of control and high-Fe plants. Data are mean ± SEM (n=3). Asterisks indicate statistical significant differences calculated by *t*-test (*, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively). Gene-specific primers are listed in **Supplemental Table S8**.

High Fe supply boost defense-related gene expression during *M. oryzae* infection

We investigated pathogen-induced transcriptional changes in leaves of high-Fe and control plants. RNAs were obtained from *M. oryzae*-inoculated and mock-inoculated high-Fe and control plants at 48 hours post-inoculation (hpi). The same criteria described above ($\log_2$ FC $\geq$ 0.5 or $\leq$ -0.5, FDR $\leq$ 0.01, P $\leq$ 0.05) were used to examine the transcriptional response to *M. oryzae* infection in leaves of high-Fe and control plants compared with their respective non-infected controls. Pair-wise comparisons revealed 6470 pathogen-regulated genes in high-Fe plants (3293 up-regulated; 3177 down-regulated) (**Figure 3A, B; Supplemental Table S2a**). In control plants, 5996 genes were regulated by *M. oryzae* infection (2879 up-regulated; 3117 down-regulated) (**Figure 3A,B; Supplemental Table S2b**). Considering that the same criteria were used for identification of DEGs in both comparisons (e.g. infected *vs* mock in high-Fe and infected *vs* mock in control plants), this difference could be explained because pathogen infection causes stronger induction of those genes in high-Fe plants compared with control plants. In line with this, heat map visualization of RNASeq data showed stronger induction by pathogen infection in an important proportion of genes in high-Fe plants relative to control plants (**Supplemental Figure S4**).

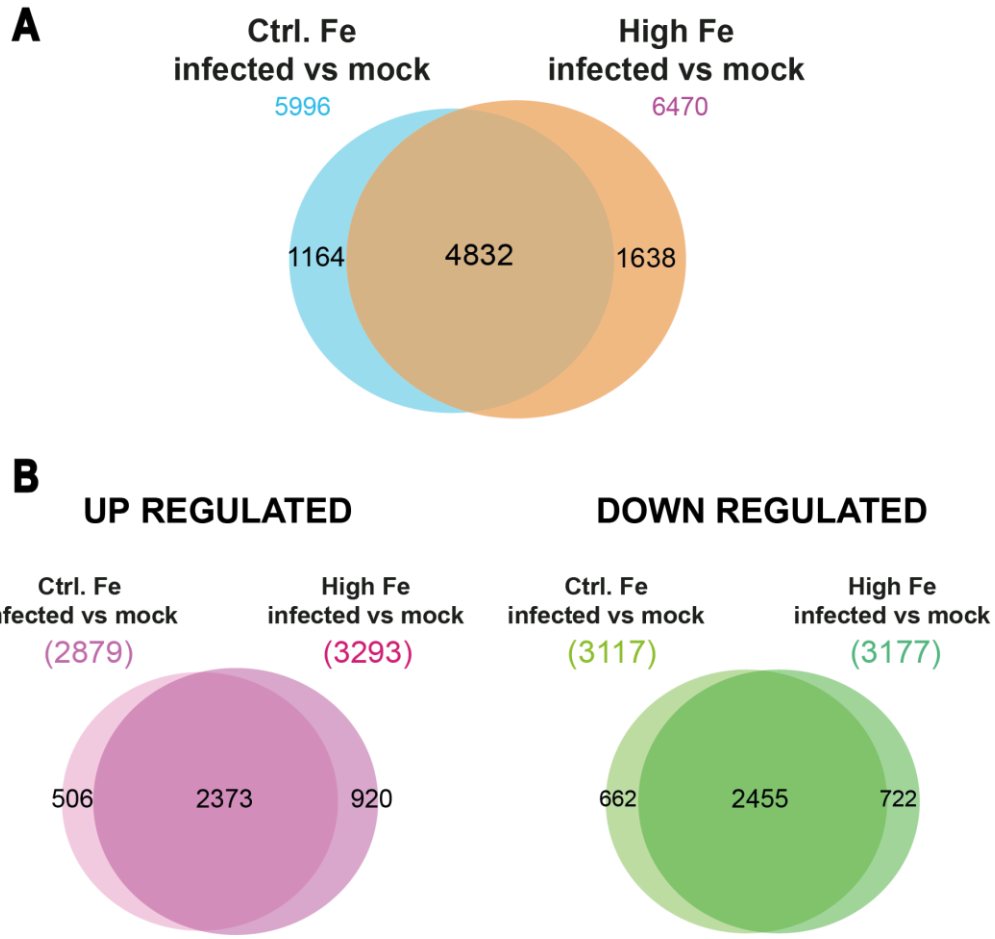


Figure 3. Response to *M. oryzae* infection in leaves of high-Fe and control plants. **(A)** Venn diagrams indicate the number of genes that are specifically and commonly regulated by *M. oryzae* infection in each Fe condition at 48 hpi. **(B)** Venn diagrams of up-regulated and down-regulated genes in each Fe condition (log_2 fold change >0.5 or <-0.5; p-value ≤ 0.05).

Gene Ontology (GO) enrichment analysis was used to identify GO annotations in Biological Processes and Molecular Function associated to the response to *M. oryzae* infection in high-Fe and control plants. The REVIGO tool was used to remove redundant GO terms (Supek *et al.* 2011; http://revigo.irb.hr). Of interest, the most abundant subcategories in Biological Processes, in both high-Fe and control plants were "Diterpene Phytoalexin Metabolism" followed by "Secondary Metabolism" and "Defense Responses" (**Figure 4A; Supplemental Table S3A and S3B**). Another GO category highly represented in up-regulated was "Phosphorus Metabolism" (**Figure 4A**) which was consistent with the observed over-representation of the "ATP binding" and "Protein kinase activity" annotations in the Molecular Function category in high-Fe and control plants (**Figure 4B**). Also in the category of Molecular Function, genes involved in "Iron binding" and "Signaling receptor activity" were over-represented in genes that are up-regulated by infection in high-Fe and control plants, while genes with "Chitinase activity" were over-represented in high-Fe plants, but not in control plants (**Figure 4B**). Regarding genes down-regulated by *M. oryzae* infection in Biological processes, they categorized in "Photosynthesis" and "Pigment biosynthetic processes", whereas in Molecular function, there were genes involved in "Iron-sulfur cluster binding" and "Zinc ion binding" (in both high-Fe and control conditions) (**Supplemental Figure S5**). Taken together, these results revealed similar GO terms associated to the response to *M. oryzae* infection in high-Fe and control rice plants.

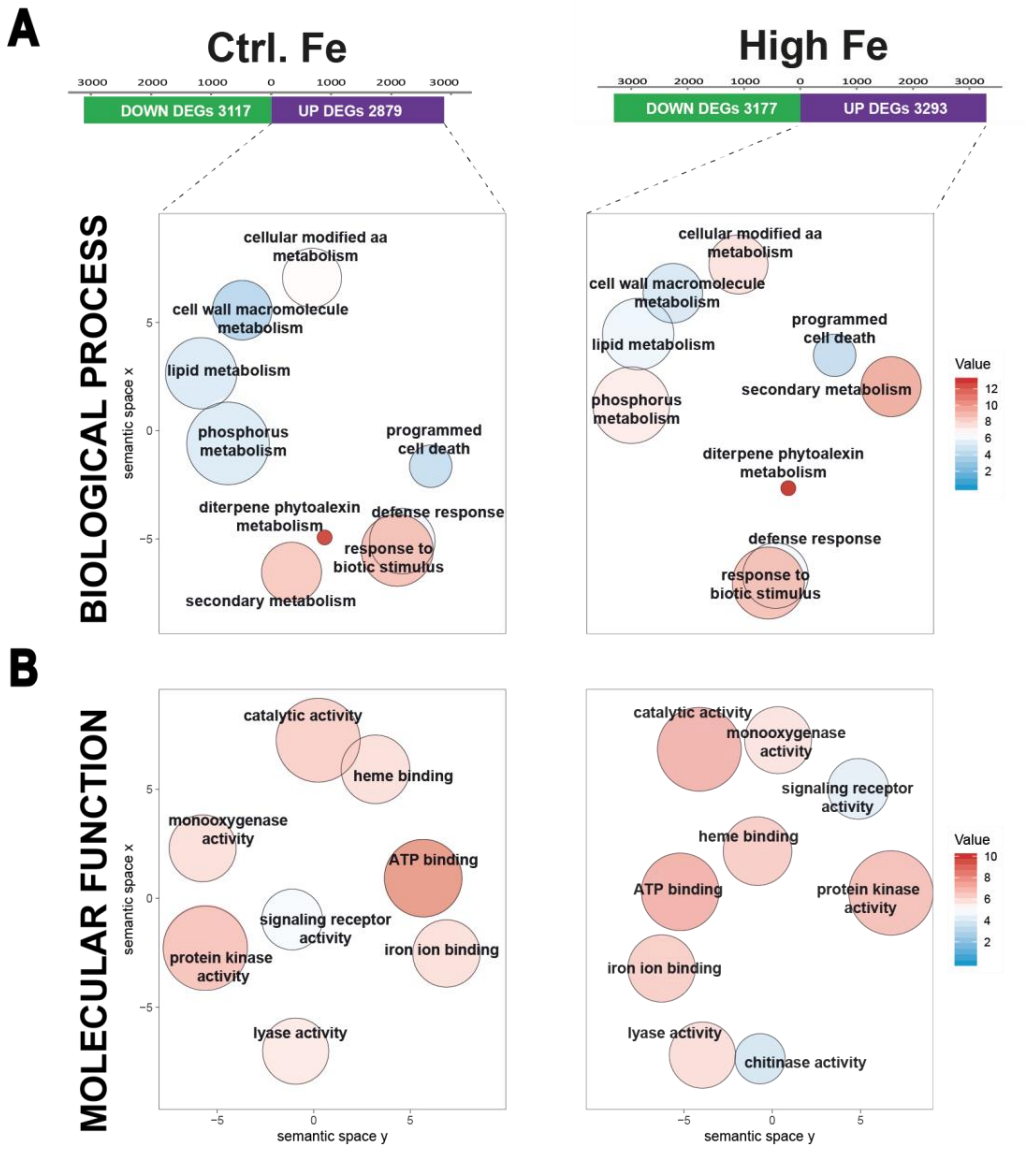


Figure 4. GO enrichment analyses of genes up-regulated by *M. oryzae* infection in control and high-Fe plants (48 hpi) in the categories of Biological Processes **(A)** and Molecular Function **(B)** GO terms were visualized using REVIGO (https://revigo.irb.hr/) after reducing redundancy and clustering of similar GO terms in the *O. sativa* database. GO terms are represented by circles and are clustered according to semantic similarities (more general terms are represented by larger size circles, and adjoining circles are most closely related). Circle size is proportional to the frequency of the GO term, whereas color indicates the enrichment derived from the AgriGO analysis (red higher, blue lower). Full data sets of DEGs and lists of GO terms are presented in **Supplemental Table S2 and S3**, respectively).

Enhanced defense responses in iron-treated rice plants

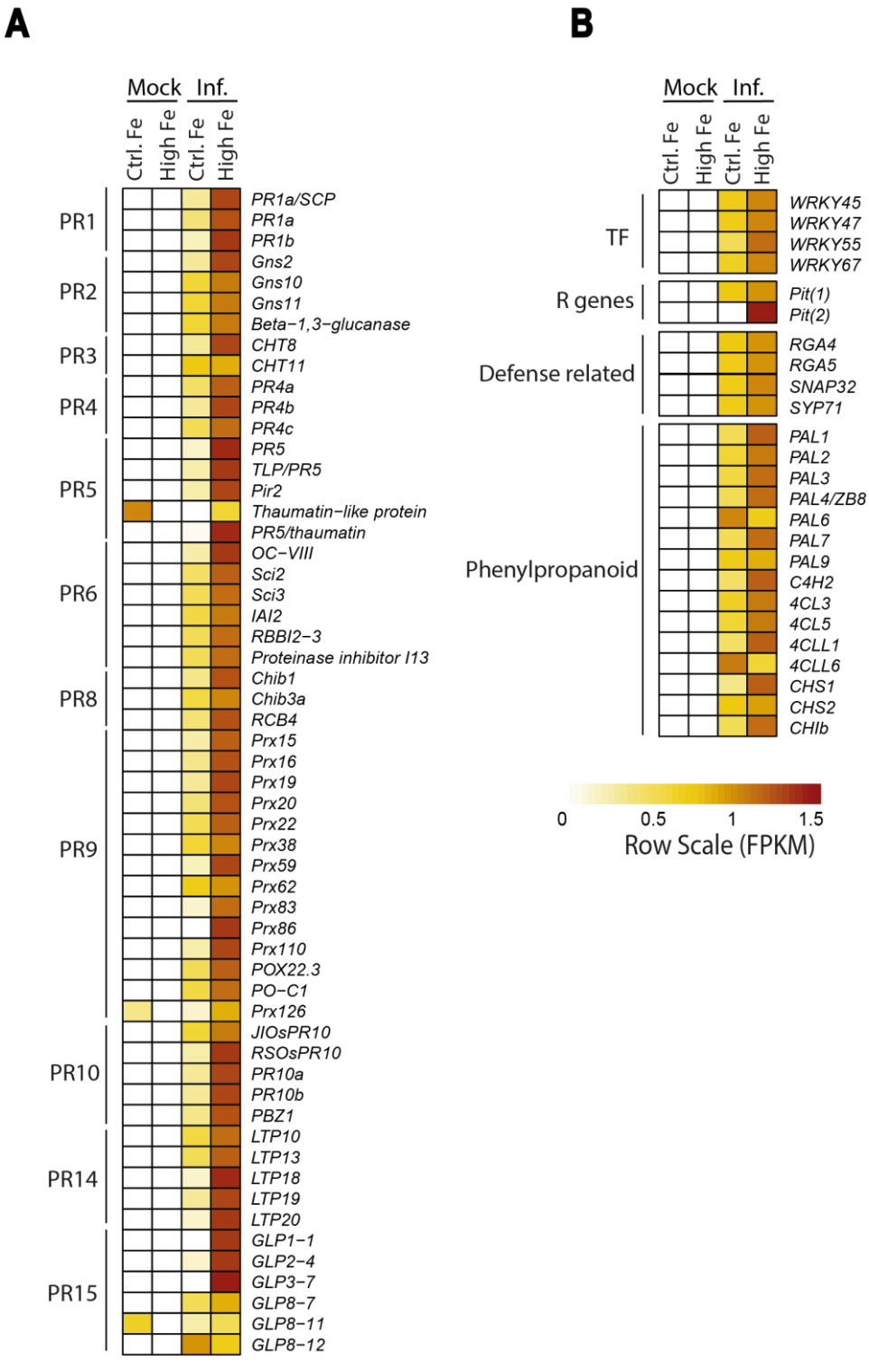
Pathogen-induced changes in defense-related gene expression in high-Fe and control rice plants were compared. As expected, *M. oryzae* infection activated the expression of *PR* genes belonging to different *PR* families in both high-Fe and control plants (**Figure 5A; Supplemental Table S4**). They included *PR1*, *PR2* (β-1,3 glucanases), *PR3*, *PR4* and *PR8* (chitinases), *PR5* (thaumatin-like proteins, TLPs), *PR6* (proteinase inhibitors), *PR9* (peroxidases), *PR10* (ribonuclease-like proteins), *PR14* (lipid transfer proteins, LTPs) and *PR15* (germin-like proteins). Among them, *OsPR1* and *OsPBZ1* (*PR10* family) are routinely used as marker genes for the induction of the rice response to *M. oryzae* infection (Agrawal *et al.* 2001; Midoh and Iwata 1996). Of interest, upon pathogen infection, *PR* genes were found to be activated to a greater extent in high-Fe plants compared with control plants (**Figure 5A; Supplemental Table S4**). It is well documented that PR proteins play an important role in plant defense against pathogen infection, and that overexpression of *PR* genes confers resistance to *M. oryzae* infection in rice (Jain and Khurana 2018; Wu *et al.* 2016). The antimicrobial activity of several PR proteins has been demonstrated (i.e. chitinases, thaumatins, LTPs, among others). Results obtained by RNA-Seq analysis were validated by qRT-PCR analysis of selected *PR* genes (*OsPR1a*, *OsPR1b*, *OsPBZ1*, a *PR10* family member, JI*OsPR10*, and *ROsPR10*) (**Supplemental Figure S6**). Thus, stronger induction of *PR* genes appears to occur in high-Fe plants compared with control plants.

RNA-Seq analysis also revealed regulation of peroxidase genes by *M. oryzae* infection (up to 14 peroxidase genes; *Prx*, *PR9* family), most of them being induced at a higher level in high-Fe plants than in control plants (**Figure 5A; Supplemental Table S4**). Peroxidases catalyze oxidation of various substrates concomitant with the decomposition of H_2O_2. It is tempting to assume that *M. oryzae*-regulated peroxidases contribute to maintain proper H_2O_2 accumulation in high-Fe plants to avoid toxic effects to the plant cell. We also noticed that *OsWRKY45*, a

positive regulator of blast resistance was induced at a higher level in high-Fe plants relative to control plants (**Figure 5B; Supplemental Table S4**).

We also noticed that genes involved in the general phenylpropanoid pathway were induced to a different extent by Fe treatment; most of them being more strongly induced in high-Fe plants compared with control Fe plants. Among them, there were *phenylalanine ammonia lyase* (*PAL*), *cinnamic acid 4-hydroxylase* (*C4H*); *4-coumarate:CoA ligase* (*4CL*), as well as *chalcone synthase* (*CHS*) and *chalcone isomerase* (*CHI*) (**Figure 5B; Supplemental Table S4**). The steps in which these genes participate in the phenylpropanoid biosynthetic pathway are indicated in **Supplemental Figure S7**. Defensive functions of phenylpropanoid compounds have long been recognized, these metabolites conferring broad-spectrum disease resistance in different plant species (Yadav *et al.* 2020). Furthermore, treatment with high Fe was associated with stronger induction of diterpenoid phytoalexin genes (see below). Altogether, there results indicated that there is a correlation between the expression level of defense-related genes (e.g. higher induction in response to infection) and the increased resistance to *M. oryzae* that is observed in high-Fe plants.

Figure 5. Expression of genes involved in the rice defense response to pathogen infection in leaves of control and high-Fe plants (48 hpi). Heat maps showing the expression level of DEGs. Left panel, gene expression is represented as row scaled FPKM from pale yellow (less expressed) to brown (more expressed). **(A)** Expression of *Pathogenesis-Related* (*PR*) genes. **(B)** Expression of defense-related genes. TF, transcription factors, R, Resistance genes. The gene name, FPKM and fold change values are indicated in **Supplemental Table S4.**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High Fe
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High Fe
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PR3
PR4
PR5
PR6
PR8
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PR14
PR15
PR1a/SCP
PR1a
PR1b
Gns2
Gns10
Gns11
Beta−1,3−glucanase
CHT8
CHT11
PR4a
PR4b
PR4c
PR5
TLP/PR5
Pir2
Thaumatin−like protein
PR5/thaumatin
OC−VIII
Sci2
Sci3
IAI2
RBBI2−3
Proteinase inhibitor I13
Chib1
Chib3a
RCB4
Prx15
Prx16
Prx19
Prx20
Prx22
Prx38
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Prx62
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Prx126
JIOsPR10
RSOsPR10
PR10a
PR10b
PBZ1
LTP10
LTP13
LTP18
LTP19
LTP20
GLP1−1
GLP2−4
GLP3−7
GLP8−7
GLP8−11
GLP8−12
B
Mock
Inf.
Ctrl. Fe
High Fe
Ctrl. Fe
High Fe
TF
R genes
Defense related
Phenylpropanoid
WRKY45
WRKY47
WRKY55
WRKY67
Pit(1)
Pit(2)
RGA4
RGA5
SNAP32
SYP71
PAL1
PAL2
PAL3
PAL4/ZB8
PAL6
PAL7
PAL9
C4H2
4CL3
4CL5
4CLL1
4CLL6
CHS1
CHS2
CHIb
0
0.5
1
1.5
Row Scale (FPKM)

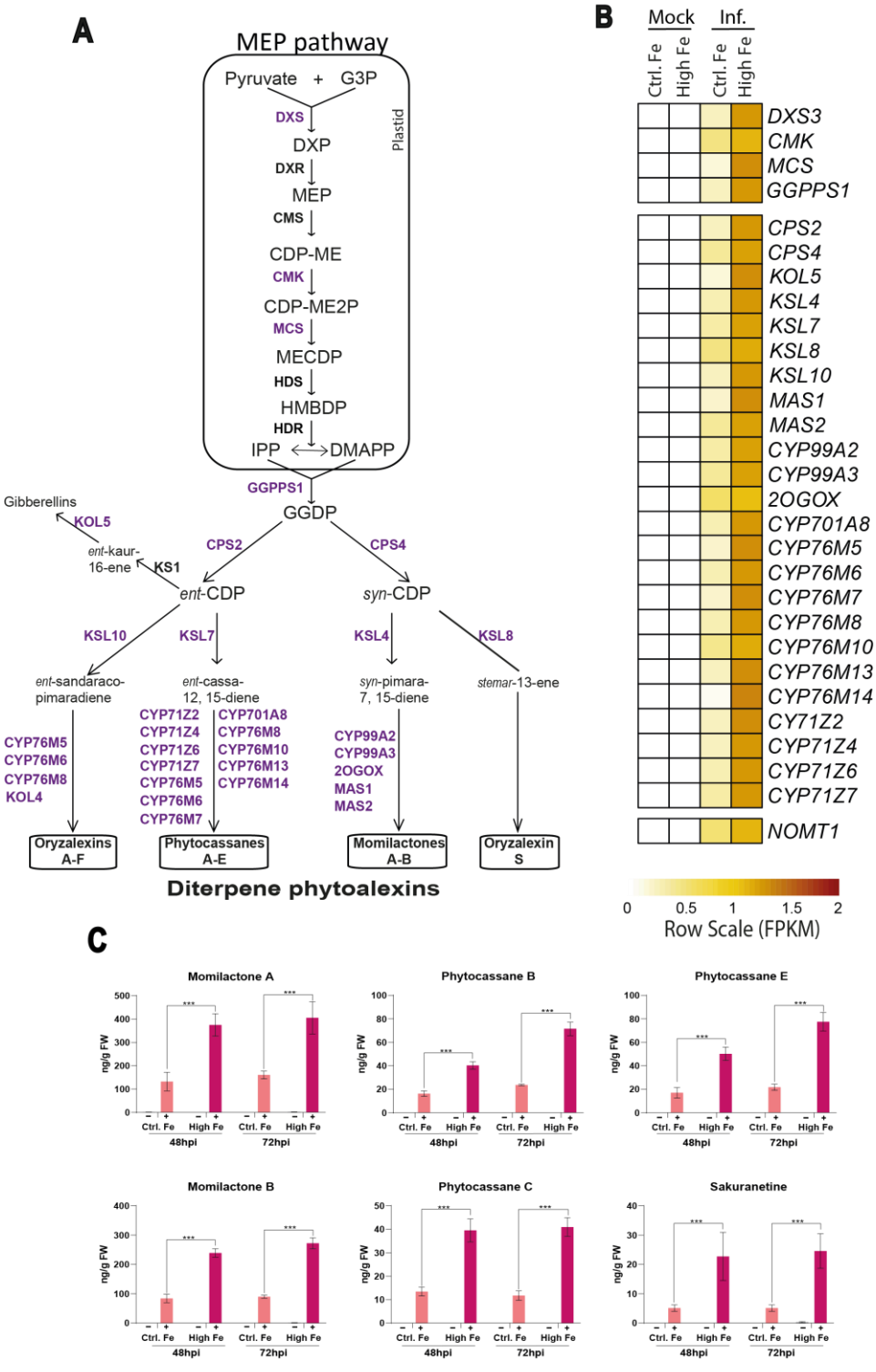
Effect of treatment with high Fe on the accumulation of rice phytoalexins

Phytoalexins are produced and accumulate in plants in response to pathogen infection, also in rice plants infected with the rice blast fungus *M. oryzae* (Duan *et al.* 2014). As previously mentioned, "Diterpenoid phytoalexin metabolism" was identified as the most enriched term in the set of genes that are up-regulated by *M. oryzae* infection in both high-Fe and control plants (see **Figure 4**). The major rice phytoalexins are diterpenoid phytoalexins and sakuretin, the only flavonoid phytoalexin so far identified in rice. Regarding diterpenoid phytoalexins, they are synthesized from the precursor molecule geranylgeranyl diphosphate (GGDP), the end product of the methylerythritol phosphate (MEP) pathway (**Figure 6A**). A heat map showing the expression of genes implicated in the MEP and diterpene phytoalexin biosynthesis pathways in response to *M. oryzae* infection in control and high-Fe plants is presented in **Figure 6B**. As expected, diterpenoid phytoalexin biosynthesis genes are not expressed in non-infected rice plants. Upon pathogen infection, the expression of genes involved in the production of major diterpene phytoalexins, namely oryzalexins A to F, phytocassanes A to E, momilactones A and B, and oryzalexin S was induced in both control and high-Fe plants. Notably, these genes were induced to a greater extend in high-Fe plants than in control plants (**Figure 6B; Supplemental Table S5**). Results obtained by RNA-Seq analysis were validated by qRT-PCR analysis of selected MEP and diterpenoid phytoalexin biosynthetic genes, namely *OsDXS3* (MEP pathway) and *OsCPS2*, *OsCPS4*, *OsMAS1*, *OsCYP701A8* and *OsCYP76M6* (diterpene phytoalexin pathway) (**Supplemental Figure S8**). Not only genes directly involved in diterpene phytoalexin biosynthesis, but also genes in the upstream methylerythritol phosphate (MEP) pathway leading to the precursor of diterpene phytoalexins GGDP (e.g. *OsDXS3*, *OsCMK*, *OsMCS* and *OsGGPPS1*) showed a higher induction in high-Fe plants compared with control plants (**Figure 6A, B; Supplemental Table S5**).

As for the only flavonoid phytoalexin in rice, sakuranetin, it is synthesized from naringenin (phenylpropanoid pathway; see **Supplemental Figure S7**) by the activity of naringenin7-O-methyltransferase (NOMT). Our RNA-Seq analysis revealed higher expression of *OsNOMT1* in response to *M. oryzae* infection in high-Fe plants compared with control plants (**Figure 6**; **Supplemental Table S5**).

Knowing that the expression of phytoalexin biosynthesis genes was strongly induced by *M. oryzae* infection in high-Fe plants, it was of interest to examine phytoalexin content in these plants. As shown in **Figure 6C**, the accumulation of diterpenoid phytoalexins (Momilactone A and B, Phytocassane B, C and E) and sakuranetin drastically increased in *M. oryzae*-infected leaves. Phytoalexin accumulation was higher in high-Fe plants than in control plants, which is consistent with RNASeq data.

Together, these results indicated that blast resistance in high-Fe plants might result, as least in part, from supereractivation of phytoalexin biosynthesis genes, and subsequent accumulation of phytoalexins.


A
MEP pathway
Pyruvate + G3P
Plastid
DXS
DXP
DXR
MEP
CMS
CDP-ME
CMK
CDP-ME2P
MCS
MECDP
HDS
HMBDP
HDR
IPP ⟷ DMAPP
GGPPS1
GGDP
Gibberellins
KOL5
ent-kaur-16-ene
KS1
CPS2
CPS4
ent-CDP
syn-CDP
KSL10
KSL7
KSL4
KSL8
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CYP76M13
CYP76M14
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MAS2
Oryzalexins A-F
Phytocassanes A-E
Momilactones A-B
Oryzalexin S
Diterpene phytoalexins
B
Mock
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High Fe
Ctrl. Fe
High Fe
DXS3
CMK
MCS
GGPPS1
CPS2
CPS4
KOL5
KSL4
KSL7
KSL8
KSL10
MAS1
MAS2
CYP99A2
CYP99A3
2OGOX
CYP701A8
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CYP76M7
CYP76M8
CYP76M10
CYP76M13
CYP76M14
CY71Z2
CYP71Z4
CYP71Z6
CYP71Z7
NOMT1
0
0.5
1
1.5
2
Row Scale (FPKM)
C
Momilactone A
Phytocassane B
Phytocassane E
Momilactone B
Phytocassane C
Sakuranetine
ng/g FW
Ctrl. Fe
High Fe
48hpi
72hpi

Figure 6. Expression of genes involved in phytoalexin biosynthesis and accumulation of phytoalexins in leaves of rice plants that have been grown under high Fe supply. **(A)** Methylerythritol phosphate (MEP) and diterpenoid phytoalexin biosynthesis pathways in rice. Genes whose expression is up-regulated by *M. oryzae* infection in control and high-Fe plants are indicated in purple color. The full name and details on the expression of these genes can be found in **Supplemental Table S5**. **(B)** Heat map showing the expression level (row scaled FPKM) at 48hpi. Left panel, gene expression is represented from pale yellow (less expressed) to brown (more expressed). **(C)** Accumulation of diterpenoid phytoalexins, momilactone A and B, phytocassane B, C, and E and the flavonoid phytohormone sakuretine in leaves of control and high-Fe plants, mock (-) and *M. oryzae*-infected (+) plants (48 and 72 hpi). FW, fresh weight. Four biological replicates (three technical replicates each) were analyzed. Data are mean ± SEM. Asterisks indicate statistical significant differences calculated by two-way ANOVA (*** $p \leq 0.001$). **G3P**, glyceraldehyde-3-phosphate; **DXP**, 1-deoxy-D-xylulose 5-phosphate; **MEP**, 2- C-methyl-D-erythritol 4-phosphate; **CDP-ME**, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; **CDP-ME2P**, 2-phospho-4-(cytidine 5'- diphospho)-2-C-methyl-D-erythritol; **MECDP**, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; **HMBDP**, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate; **IPP**, isopentenyl diphosphate; **DMAPP**, dimethylallyl diphosphate; **GGDP**, geranylgeranyl diphosphate; and **CDP**, copalyl diphosphate.

Pathogen infection has an impact on the expression of iron homeostasis genes and iron content in rice leaves

The observation that *M. oryzae* infection provokes alterations in Fe^{3+} accumulation in distinct cells of the infected leaves (i.e. cells adjacent to the invaded cells; see **Figure 1C**) pointed to a regulation of genes involved in iron homeostasis in the *M. oryzae*-infected rice leaves. Indeed, RNASeq analysis revealed that a large number of genes with a known function in iron homeostasis are regulated by pathogen infection. Although many of these genes showed a similar trend in their response to infection in high-Fe and control plants, the intensity of the response varied depending on the Fe condition (e.g. stronger induction or repression by pathogen infection) (**Figure 7A,** control, infected vs mock; high-Fe, infected vs mock; **Supplemental Table S6**). Genes that are repressed by *M. oryzae* infection included genes encoding the transcription factors *OsIRO2*, *OsIRO3*, *OsIMA1*, *OsIMA2*, *OsHRZ1*, *OsHRZ2*, or the oligopeptide transporter *OsOPT7*, whose expression is induced in response to Fe deficiency (Bashir *et al.* 2015; Ogo *et al.* 2017; Kobayashi *et al.* 2013; Wang *et al.* 2020) (**Figure 7A; Supplemental Table S6**)

As previously mentioned, excess Fe might become toxic to the plant via the Fenton reaction in which Fe^{2+} reacts with H_2O_2 to produce the hydroxyl radical (·OH), the most reactive ROS species. Although Fe^{3+} also reacts with H_2O_2 (Fenton-like reaction) to produce Fe^{2+} (as a source for subsequent production of toxic radicals in the Fenton reaction), the reaction of Fe^{3+} with H_2O_2 is substantially slower than the reaction of H_2O_2 with Fe^{2+}. Inside the cell, the availability of free Fe^{2+} must be tightly controlled to avoid the Fenton's reaction to occur. Storing Fe and/or Fe partitioning into sub-cellular compartments, mainly apoplast and vacuoles, are crucial to alleviate Fe toxicity (Moore *et al.* 2014). Ferric Reductase Oxidase (FRO) enzymes play an important role in maintaining proper Fe intracellular distribution and transport between the cytoplasm and the vacuole (Li *et al.* 2019). FRO is encoded by two genes in the rice genome (*OsFRO1* and *OsFRO2*). Of them, *OsFRO1* was reported to be responsible of the reduction of ferric Fe (Fe^{3+}) into ferrous Fe (Fe^{2+}) in the vacuole, which makes the vacuolar Fe available to the cytoplasm through Fe^{2+} transporters. Both *OsFRO1* and *OsFRO2* were found to be repressed by *M. oryzae* infection in control and high-Fe rice plants (**Figure 7**). Under high Fe conditions, downregulation of *OsFRO1* would reduce the amount of Fe^{2+} available for cytoplasm which would alleviate toxic effects caused by Fe excess. In the plant body, free Fe^{2+} is toxic; therefore, iron transport requires formation of complexes with phytosiderophores, such as the iron quelators of the mugineic acid (MA) family of phytosiderophores. To note, upon pathogen challenge, key genes related to the synthesis of MAs (*OsSAM1*, *OsSAMS2*, *OsDMAS1*) and phytosiderophore efflux transporters (*OsPEZ1*, *OsTOM3*) were induced at a higher level in high-Fe than in control plants.

On the other hand, *M. oryzae* infection strongly induced *FERRITIN2* (*FER2*) expression in high-Fe plants, and at a lesser extent also in control plants (**Figure 7A, Supplemental Figure S9**). Ferritin is the major iron-storage protein considered essential for tolerance to excess Fe. Its ability to sequester iron gives ferritin the function of protection against oxidative stress by mitigating damage from the Fenton reaction. In other studies, it was described that ferritins are up-regulated in various plant species following infection (Mata *et al.* 2001).

The NRAMP family of metal transporter proteins is of particular importance in iron compartmentalization which can be useful for Fe homeostasis, especially in conditions of excess of this element. RNASeq analysis showed that 4 genes encoding NRAMP proteins (Natural Resistance-Associated Macrophage Proteins) showed regulation by *M. oryzae* infection in control and high-Fe plants (**Figure 7A**). They were: *OsNramp1, OsNramp3, OsNramp5, OsNramp6 and OsNramp7* (all plasma membrane proteins). RT-qPCR analysis confirmed regulation of iron homeostasis-related genes in high-Fe and control plants *(OsIRO2, OsIRO3, OsFRO1, OsFRO2, OsNRAMP1, OsFER2)* (**Supplemental Figure S9**).

Other Fe-responsive genes whose expression is regulated by *M. oryzae* infection in rice leaves were members of the *Yellow Stripe-Like* (*YSL*) family of metal-NA (nonproteinogenic amino acid nicotianamine) transporters and zinc-regulated transporter ITR-like proteins (ZIP) family, including *OsYSL5*, *OsYSL6*, *OsYSL9*, *OsYSL12*, *OsYSL13*, *OsYSL14* and *OsYSL15* (among YSLs), and *OsZIP2*, *OsZIP4* and *OsZIP8* from the ZIP family (**Figure 7A**). NA was reported to be an indispensable component for the intracellular and intercellular distribution of iron in plants (Ishimaru *et al.* 2010). Regarding ZIP genes, although their expression is regulated by zinc (i.e. *OsZIP4),* crosstalk between Fe and Zn homeostasis is known to occur in plants (Ishimaru *et al.* 2005).

Altogether RNASeq analysis revealed that *M. oryzae* infection triggers important transcriptional alterations in the expression of genes involved in Fe homeostasis. A differential regulation of these genes in high-Fe plants compared with control plants (e.g. stronger or weaker transcriptional responses) and spatial regulation of their expression in cells of the infected leaves might be crucial not only to arrest pathogen infection but also to alleviate Fe toxicity in the host plant.

Having established that pathogen infection regulates the expression of iron-associated genes, we examined Fe content in leaves of high-Fe and control plants, under infection and non-infection conditions (24 hpi). As expected, treatment with high Fe results in an increase in total Fe content (**Figure 7B)**. Upon pathogen challenge, there was a small but significant increase in total Fe content in leaves of control plants, but not

in high-Fe plants (**Figure 7B**). To gain more information about effect of pathogen infection on iron content in rice leaves, we measured apoplastic Fe levels in *M.oryzae*-inoculated and mock-inoculated control and high-Fe plants. As shown in **Figure 7C**, *M. oryzae* infection is accompanied by a decrease in apoplastic Fe both in control and high-Fe plants, supporting perturbation of iron distribution in rice leaves during blast infection (e.g. cellular and apoplastic iron distribution). The concentration of apoplastic Fe in *M. oryzae*-infected high-Fe plants was similar to that in non-infected control plants.

Finally, since iron ions show unique reactivity depending on its redox state (e.g. Fe^{2+} or Fe^{3+}), we investigated the oxidation states of Fe accumulating in *M. oryzae*-infected leaves of high-Fe and control plants which was then compared with that in the non-infected leaves for each condition. The ferrozine-based assay was used to quantify Fe^{3+} and Fe^{2+} content (Chen and Lewin 1972). In the absence of pathogen infection, treatment with high Fe increased both Fe^{3+} and Fe^{2+} levels (**Figure 7D**, grey and black bars). Upon pathogen challenge, Fe^{2+} content was not significantly altered in leaves of control or high-Fe plants (relative to the corresponding non-infected leaves). In the case of Fe^{3+}, however, while pathogen infection increased Fe^{3+} content in control plants (infected *vs* non-infected control), its level decreased in high-Fe plants (infected *vs* non-infected high-Fe) (**Figure 7D**, pink bars). Thus, Fe^{3+} content in *M. oryzae*-infected high-Fe plants were similar to those in non-infected control plants.

Collectively, results obtained on the transcriptome analysis in combination with Fe analyses point to an important regulation in the expression of genes involved in iron homeostasis in rice leaves during *M. oryzae* infection. Treatment with high-Fe causes an increase in Fe content in rice leaves, total Fe (Fe^{2+} and Fe^{3+}) and apoplastic Fe. Different response patterns are, however, observed between control and high-Fe plants during pathogen infection. Whereas in control plants pathogen infection increased the level of Fe (total Fe, and Fe^{3+}), in *M. oryzae*-infected high-Fe plants Fe content decreased (total Fe, apoplastic Fe and Fe^{3+}). A tight control of iron homeostatic mechanisms might occur during infection by *M. oryzae* in rice leaves.

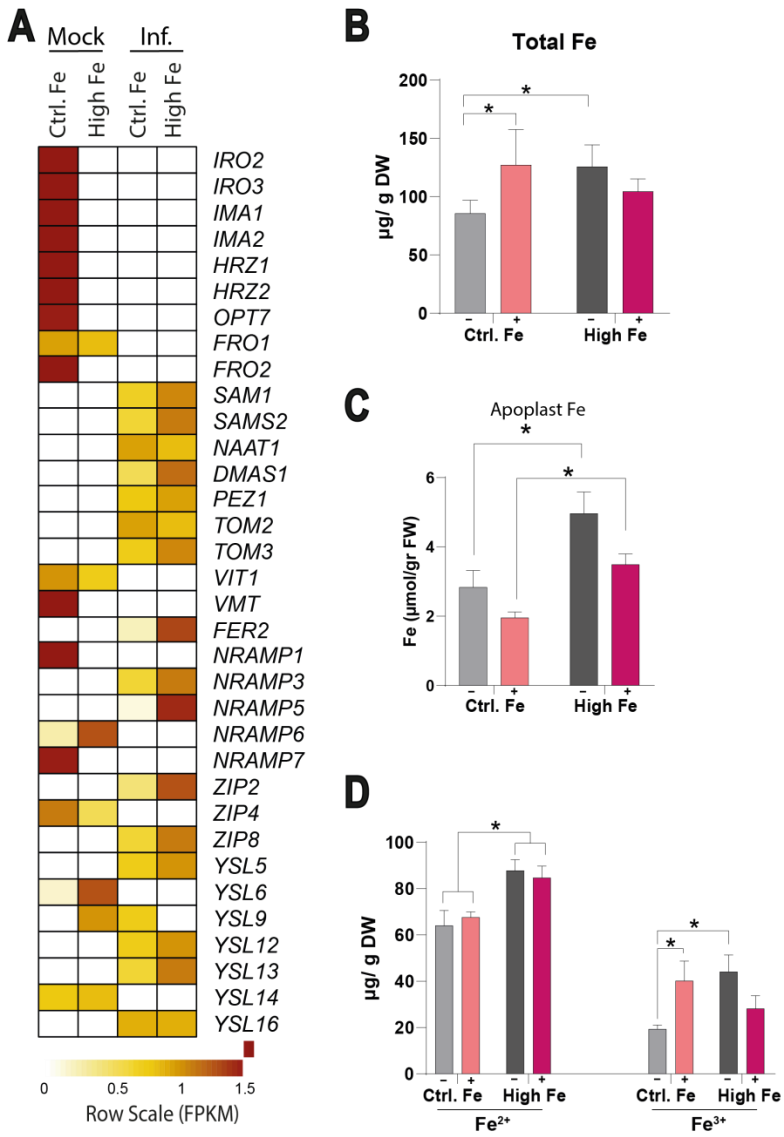

Figure 7. Expression of genes involved in Fe homeostasis and Fe accumulation in leaves of rice plants that have been grown under high-Fe supply. **(A)** Heat map showing the expression level (row scaled FPKMs) at 24 hpi. Left panel, gene expression is represented from pale yellow (less expressed) to brown (more expressed). The full name and details on the expression of these genes are indicated in **Supplemental Table S6. (B)** Total Fe content estimated by colorimetric Ferrozine method in the youngest developed leaf (third leaf) at 48 hpi. **(C)** Fe content in the apoplast of leaves (youngest developed leaves) in *M. oryzae*-inoculated (+) and mock-inoculated (-) leaves. Fe content was estimated by ICP-MS. **(D)** Fe^{2+} and Fe^{3+} content was determined by the colorimetric Ferrozine method in the youngest developed leaf of control and high-Fe plants at 24 hpi. DW, dry weight. Five independent biological replicates (two technical replicates each) were analyzed in B, C and D. Data are mean ± SEM. Asterisks indicate statistical significant differences calculated by two-way ANOVA *, $p \leq 0.05$).

DISCUSSION

In this study, we provide evidence that the iron status of rice plants greatly influences resistance to *M. oryzae*. Several lines of evidence are consistent with this conclusion. First, we show that treatment with high Fe confers resistance to infection by the rice blast fungus. Second, blast resistance in high-Fe rice plants was associated to stronger induction of defense-related genes during pathogen infection, including *PR* genes. Third, a superinduction of genes involved in phytoalexin biosynthesis occurs during infection of high-Fe rice plants. Consequently, high-Fe rice plants accumulated major rice phytoalexins, diterpene phytoalexins (momilactones A, B, phytocassanes A-E, oryzalexins A-F and oryzalexin S), and sakuranetin. Diterpene phytoalexins and sakuretin are known to accumulate in rice leaves during *M. oryzae* infection, these metabolites exhibiting antifungal activity against *M. oryzae* (Hasegawa *et al.* 2014; Okada *et al.* 2007; Sánchez-Sanuy *et al.* 2019). The observed accumulation of phytoalexins might contribute, at least in part, to the phenotype of blast resistance in high-Fe plants.

Plants have evolved multifaceted strategies to avoid adverse consequences of Fe deficiency and excess in order to maintain normal growth and development. Consistent with an increase in total Fe content in Fe-treated plants, genes that typically induced by Fe deficiency were found to be repressed in high-Fe plants. Under the experimental conditions assayed in this work, however, Fe treatment had a low impact on the leaf transcriptome. Of interest, major differences between control and Fe-treated plants were observed during pathogen infection. Here, high-Fe plants developed a more robust defense response than control plants, which is in agreement with the phenotype of blast resistance that is observed in these plants. Superactivation of defense-related genes in high-Fe rice plants is reminiscent of defense priming, an adaptive strategy that improves the defensive capacity of plants (Gourbal *et al.* 2018; Martinez-Medina *et al.* 2016). In addition to *PR* genes and phytoalexin biosynthetic genes, our transcriptomic data showed stronger induction of *OsWRKY45*

in high-Fe plants than control plants in response to *M. oryzae* infection. In other studies, an *OsWRKY45*-dependent priming of diterpenoid phytoalexin biosynthesis genes was described, while *OsWRKY45* overexpression conferred resistance to the rice blast fungus in rice (Akagi *et al.* 2014). Moreover, *OsFER2* was strongly up-regulated after pathogen infection in Fe-treated rice plants. Ferritin is known to bind excess Fe in a safe and bioavailable form to avoid cellular Fe toxicity (Aung and Masuda 2020). The observation that *Ferritin* is regulated by Fe treatment as well as by *M. oryzae* infection supports that plants have evolved mechanisms to sustain Fe homeostasis and immune reactions in concerted action. In other studies, ferritin was implicated in plant responses to pathogen infection (Dellagi *et al.* 2005; Herlihy *et al.* 2020).

Considering the outstanding importance of Fe in the generation of ROS, and that ROS mediate the induction of defense responses, we investigated Fe and ROS accumulation in high-Fe rice plants that have been inoculated with *M. oryzae* spores. Dual histochemical staining revealed that both Fe and H_2O_2 accumulate at the sites where pathogen penetration occurs. This localized accumulation of Fe might well trigger ROS production for the activation of immune responses to arrest pathogen invasion in high-Fe rice plants. The possibility of transfer of Fe from distal, non-infected leaf regions towards the infection sites should be also considered. If so, changes in the expression of iron homeostasis genes in the fungal-infected leaves would facilitate recruitment of Fe towards the infection sites. In addition to Fe recruitment, iron released from the first invaded dead cells might also provoke oxidative stress, thus contributing to immunity. The observed alterations in Fe distribution and subsequent accumulation of ROS in high-Fe plants is consistent with ferroptotic cell death at the sites of pathogen penetration, a response that was recently described in rice plants infected with an avirulent *M. oryzae* strain (Dangol *et al.* 2019; Distéfano *et al.* 2021). Dangol and collaborators (2019) also described that infection with a virulent strain of *M. oryzae* did not induce accumulation of Fe, thus, resulting in a phenotype of susceptibility. It is tempting to hypothesize that during

infection of Fe-treated rice plants with a virulent *M. oryzae* isolate (present work), the rice plant develops a response similar to that in non-treated plants upon infection with an avirulent *M. oryzae* strain (Dangol *et al.* 2019), by accumulating Fe and ROS at the sites of infection.

Without infection, higher levels of Fe (total Fe, apoplastic Fe, Fe^{2+} and Fe^{3+}) were observed in high-Fe plants compared with control plants. Upon pathogen challenge, total Fe increased in control plants while slightly decreasing in high-Fe plants. The observed reduction in total Fe content in high-Fe plants in response to pathogen infection might be the consequence of a reduction in apoplastic Fe and/or Fe^{3+} in these plants.

Being a foliar pathogen, *M. oryzae* entirely depends on the host tissue to acquire nutrients required for pathogen growth, including Fe. As both partners, host and pathogen, compete for Fe, a tight control over Fe homeostasis is of central importance in determining the outcome of the interaction. In this multifaceted process, the host plant might create conditions that restrict fungal proliferation in the plant tissue. Thus, the rice plant might deploy strategies to sequester Fe away from the pathogen during infection, a response that has been observed in other plant species (Herlihy *et al.* 2020). There is then the possibility that the observed reduction in total Fe and Fe^{3+} content that occurs in high-Fe plants during *M. oryzae* infection (which does not occur in fungal-infected control plants) might represent a limiting factor for fungal growth in high-Fe plants. Also, from results here presented, localized accumulation of Fe at the sites of pathogen infection, and subsequent accumulation of ROS, would provoke stronger induction of defense mechanisms in high-Fe rice plants, while increased ROS can be potentially toxic to the pathogen. On the other hand, the pathogen might use strategies for Fe acquisition from the host plant for its own nutritional needs, such as the production of siderophores (Hof *et al.* 2009). Also, when considering the influence that the Fe status in the host plant might have on the pathogen, it should be taken into account that the effect might depend on the pathogen lifestyle (necrotroph, biotroph, hemibiotroph pathogens). It will then be of great

interest to investigate whether treatment with Fe confers resistance to pathogens with different lifestyles in rice.

Several studies have gathered evidence for a cross-talk between Fe signaling in Fe-starved plants and immunity (Aznar *et al.* 2015; Herlihy *et al.* 2020; Kieu *et al.* 2012; Verbon *et al.* 2017). For instance, Fe deficiency was found to confer resistance to *D. dadantii* and *B. cinerea* in Arabidopsis (Kieu *et al.* 2012). In other studies, Fe-starved maize was unable to produce ROS during infection with *Colletotrichum graminicola* which correlated with increased susceptibility to this fungal pathogen (Ye *et al.* 2014). These authors also described that maize recruits Fe^{3+} to the *Blumeria graminis* infection sites (Ye *et al.* 2014). Wheat plants infected with *B. graminis* f.sp. *tritici*, accumulated Fe^{3+} at cell wall appositions to mediate an oxidative burst (Liu *et al.* 2007). Results here presented on rice plants treated with high Fe, together with those previously reported by Dangol and collaborators (2019) on rice plants infected with an incompatible *M. oryzae* strain, demonstrated that Fe accumulates at the sites of fungal infection. From the information gained in maize, wheat and rice plants, it appears that accumulation of Fe, and subsequent ROS production, might be a critical response to arrest pathogen infection in cereal species. As most studies on the Fe nutritional status and immunity have been carried out in plants under Fe-limiting conditions, the main challenge now is to elucidate how iron homeostasis is controlled in leaves of rice plants under high Fe supply, and how infection by a foliar pathogen modulates these processes.

CONCLUSIONS

In conclusion, the results presented here demonstrated that Fe plays a critical role in regulating immune responses in rice, and further supports cross-talk between Fe signaling and immunity in rice plants. Protection against *M. oryzae* infection can be explained by a localized accumulation of Fe at the sites of pathogen penetration. A tight regulation of Fe distribution in the infected leaves would then contribute to blast resistance. The mechanistic connection between accumulation of Fe and a successful immune response is explained at least in part by the iron's capacity to produce ROS. A better understanding on the mechanisms underlying Fe homeostasis in leaves will allow the development of appropriate strategies for protection of rice against the blast fungus. Our observations provide an important basis for future investigation into the molecular mechanisms underlying Fe homeostasis in rice plants treated with high Fe.

MATERIALS AND METHODS

Plant and fungal material

Rice plants were grown at 28 °C with a 14 h/10 h light/ dark cycle. For Fe treatment, plants were grown in soil (turface:vermiculite:quartz sand [2:1:3]), and watered with a half-strength Hoagland solution during 14 days (2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM NH_4NO_3, 25 µM KH_2PO_4, 23.15 µM H_3BO_3, 4.55 µM $MnCl_2 \cdot 4H_2O$, 0.38 µM $ZnSO_4 \cdot 7H_2O$, 0.1 µM $CuSO_4 \cdot 5H_2O$, 0.14 µM $Na_2MoO_4 \cdot 2H_2O$, 50 µM Fe-EDDHA, pH 5.5). After 14 days, the plants were supplied with the same nutrient solution containing 1 mM Fe-EDDHA or maintained at 50 µM Fe-EDDHA. After 5 days of treatment, rice plants were infected with *M. oryzae* spores as described below.

Blast resistance assays

The fungus *M. oryzae* (strain Guy-11) was grown in Complete Media Agar (CMA, 9 cm plates, containing 30 mg/L chloramphenicol) for 15 days at 28 °C under a 16 h/8 h light/dark photoperiod condition. *M. oryzae* spores were prepared as previously described (Campo *et al.* 2013). Soil-grown plants (3–4 leaf stage) were spray-inoculated with *M. oryzae* spores ($5x10^5$ spores/ml; 0.3 ml/plant) using an aerograph at 2 atm of pressure. Plants were maintained overnight in the dark under high humidity, and allowed to continue growth for the required period of time. One of three independent experiments with similar results is shown (n=10).

Phenotypical analysis and chlorophyll content

Chlorophyll content was determined using the SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies). SPAD readings were

obtained in the youngest developed leaf of rice plants grown in different Fe concentrations. Data are mean of 20 biological replicates. Shoot and root fresh weight (FW) was determined in three-week-old rice plants treated, or not, with 1 mM Fe at 5 days and 19 days after the onset of treatment. Data are mean of 10 biological replicates.

Plant tissue staining

H_2O_2 determination was carried out using H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) staining following the procedure described by Kaur *et al.* 2016. Briefly, the rice leaves were cut into pieces (3 cm) and incubated in a solution containing 10 µM H_2DCFDA, vacuum infiltrated during 5 min, and then maintained in darkness at room temperature for 10 min. The samples were washed three times with distilled water and stored in 20% glycerol and immediately visualized. The fluorescence emitted by H_2DCFDA was observed at 488 nm in an AixoPhot DP70 microscope under GFP fluorescence (488 nm excitation, 509 nm emission). Fluorescence was quantified using ImageJ software.

DAB (3,3 Diaminobenzidine) and Perls staining were carried out in mock- and *M. oryzae*-inoculated rice leaves, at 48 h post-inoculation [hpi]. For DAB staining, the rice leafs were cut in 3 cm pieces, incubated in a solution containing 1mg/ml DAB, vacuum infiltrated during 30 minutes, and then maintained in the dark at room temperature overnight. Leaves were washed with 75% ethanol.

Following DAB staining, the rice leaves were subjected to Perls staining following the procedure described by Stacey *et al.* (2008) with some modifications. Briefly, rice leaves were vacuum-infiltrated in a fixing solution (chloroform:methanol:glacial acetic acid; 6:3:1, v/v) for 1 h and incubated overnight at room temperature. After washing with distilled water (three times), samples were vacuum infiltrated with a pre-warmed (37 °C) staining solution (4% HCl and 4% K-ferrocyanide at equal volumes) for 1 h, incubated 4 h more at 37 °C in the same solution

without vacuum, and then washed three times with distilled water. Sections were mounted in glycerol 50%. All the images were taken in an AixoPhot DP70 microscope under bright light. Three biological replicates with 4 technical replicates each were performed.

RNA-seq analysis

Total RNAs were obtained using Maxwell(R) RSC Plant RNA Kit (Promega). For RNA-Seq, two biological replicates for each condition were examined, each biological replicate consisting of leaves from five individual plants (youngest totally expanded leaf). RNA concentration and purity were checked using a spectrophotometer (NanoDrop, ND-1000). RNA quality and integrity was examined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.; RNA integrity number (RIN) ≥ 8). A total of eight libraries were subjected to RNA-Seq analysis (125 paired-end reads), yielding an average of 24 812 610 clean reads/library. Raw reads were processed and analyzed as previously described (Sánchez-Sanuy *et al.* 2019). Reads were mapped against the reference genome, *Oryza sativa* sp. *japonica* (IRGSP-1.0 Ensembl release 41). To identify differentially expressed genes, a FDR cutoff < 0.01 and log2FC -0.5 ≤ or ≥ 0.5 was applied. Gene Ontology (GO) enrichment analysis (GOEA) was performed using the AgriGO web tool (Parametric Analysis of Gene Set Enrichment) (Du *et al.* 2010, https://bioinfo.cau.edu.cn/agriGOv2/). Enriched GO terms were clustered and plotted with the online analysis tool ReviGO (https://revigo.irb.hr/, Supek *et al.* 2011).

Expression analysis by qRT-PCR

Total RNA was extracted from plant tissues by using TRizol reagent (Invitrogen). For quantitative RT-PCR (RT-qPCR), the first complementary DNA was synthesized from DNase-treated total RNA

(0.5 µg) with High Capacity cDNA Reverse Transcription (Life technology, Applied Biosystems). Amplification involved cDNA (2 µl, 5 ng/µl) in optical 384-well plates (Roche Light Cycler 480; Roche Diagnostics, Mannheim, Germany) with SYBR Green I dye and gene-specific primers (**Supplemental Table S8**). The *Ubiquitin1* gene (Os06g0681400) was used to normalize transcript levels. Results from one of three independent experiments with similar results are shown. Three biological replicates and two technical replicates were analyzed. Asterisks indicate significant differences calculated by two way ANOVA *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

Analysis of apoplastic Fe

The apoplast wash fluid (AWF) was obtained from the same plant material used for the analysis of total Fe content as previously described (Kim *et al.*, 2013). Briefly, the youngest developed leaves of three-week-old plants (1 gr of fresh weight) were cut in 5 cm pieces and placed in a tube containing 30 ml of 200 mM $CaCl_2$, 5 mM Na-acetate (pH 4.3 adjusted with glacial acetic acid) and 0.1 mM TPCK and 0.1 mM PMSF (freshly prepared), and kept on ice under constant agitation for 1 hour. Then, vacuum was applied for 30 minutes and leaves were removed and carefully dried. Dried leaves were rolled and placed in a tube for AWF recovery. Leaves were centrifuged a 3000 rpm, 15 minutes at 4ºC. AWF was recovered from the bottom of the tube and kept in -20 until use.

To assess that the apoplast fluid was devoid of cytoplasmic contaminants we performed the malate dehydrogenase (MDH) activity assay. For each material, MDH activities in total protein extracts was compared with that of apoplast samples. For this, total protein extracts were prepared from rice leaves using 1 mM MOPS, pH 7.5, containing 5 mM NaCl, and protease inhibitors (TPCK and PMSF, 0.1 mM each). Protein concentrations of total and apoplastic fluid extracts was quantified using Bradford. For MDH assay, protein samples (10 µl of either total protein or

apoplastic fluid extracts, at 0.1 µg/µl) were added to the reaction mixture containing 0.4 mM NADH, 0.2 mM oxaloacetic acid, 1mM MOPS, pH 7.5, in 96 micro titer plates. The absorbance at 340nm at 25ºC was recorded during 10 minutes. MDH activity was calculated as (Δabsorbance 340.min-1)/mg). Malate Dehydrogenase (MDH) activity was found to be in the range of 3.5% - 4% compared to total leaf protein extracts (**Supplemental Figure S10**).

Fe content was determined by inductively coupled plasma-mass spectrometry (ICP–MS; Bruker AURORA M90 ICP–MS, Bruker Daltonik GmbH, Bremen, Germany). Briefly, 30 µL of the apoplastic solutions were diluted to 1.2 mL with 2% HNO3 in (v/v) bidistilled water. In order to check the nebulization performance, an aliquot of 2 mg L-1 of an internal standard solution (72Ge, 89Y, and 159Tb) was added to the samples and calibration standards to give a final concentration of 20 µg L-1. Possible polyatomic interferences were removed by using CRI (Collision–Reaction-Interface) with an H_2 flow of 80 mL min-1 flown though skimmer cone. Calibration curve were obtained using multi- and a single- (for P) ICP-MS standard solutions (Ultra Scientific, USA). Five biological replicates were analyzed. Asterisks indicate significant differences calculated by one way ANOVA *, $P \leq 0.05$; **, $P \leq 0.01$).

Quantification of Ferrous (Fe^{2+}) and Ferric Fe (Fe^{3+})

The ferrozine reagent was used for quantification of Fe^{2+}. For Fe^{3+} quantification samples were treated with hydroxylamine, a reducing agent converting Fe^{3+} to Fe^{2+} (Stookey 1970). Ferrozine, in the presence of ferrous ions gives a pink-purple color which can be measured spectrophotometrically. Fe content was determined as previously described (Adolfsson *et al.* 2017). For this, the youngest totally expanded leaves of three-week-old plants were dried (5 days at 80º C). 50-70 mg of dried samples were pulverized in a Tissue Lyser, dried material was resuspended in 1M HCl (700 µl) and kept under constant agitation

overnight. After centrifugation (10.000 rpm, 5 min, room temperature), the supernatant was recovered. Centrifugation was repeated twice. 150 µl were recovered for Fe^{2+} quantification and other 150 µl for total Fe quantification (Fe^{2+} + Fe^{3+}). For Fe^{2+} quantification 15 µl of ferrozine 2 mM diluted in ammonium acetate 5M pH 9.5 were added to 150 µl of plant extract. For total Fe quantification 75 µl of hydroxylamine 6.25 M diluted in distilled water were added to the 150 µl of plant extract and incubated for 15 minutes. Then, 15 µl of ferrozine 2 mM diluted in ammonium acetate pH 9.5 were added. Blanks were carried out following the same procedures but adding 15 µl of ammonium acetate 5M pH 9.5 to plant extracts without ferrozine. Absorbance was read at 562 nanometers. Fe concentration was estimated by subtracting ferrozine based values with blank values. Fe^{3+} concentration was estimated by subtracting reduced hydroxylamine values (total Fe) with Fe^{2+} values. To determine final Fe concentration, standard curves at increasing Fe concentrations were performed. Five biological replicates with two technical replicates were performed. Asterisks indicate significant differences calculated by two way ANOVA *, $p \leq 0.05$; **, $p \leq 0.01$)

Quantification of rice phytoalexins

For quantification of rice phytoalexins, leaf segments (aprox. 2 cm in length, 200–300 mg) were mixed with 40 vol. of 70% methanol and incubated at 4 °C overnight with constant rotation. A 1 ml aliquot was centrifuged at maximum speed to remove cell debris and used for quantification of phytoalexins.

Phytoalexins were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Miyamoto *et al.* 2016). Three biological replicates with two technical replicates each were performed. Significant differences in phytoalexin accumulation were evaluated with two way ANOVA *, $p \leq 0.05$; **, $p \leq 0.01$).

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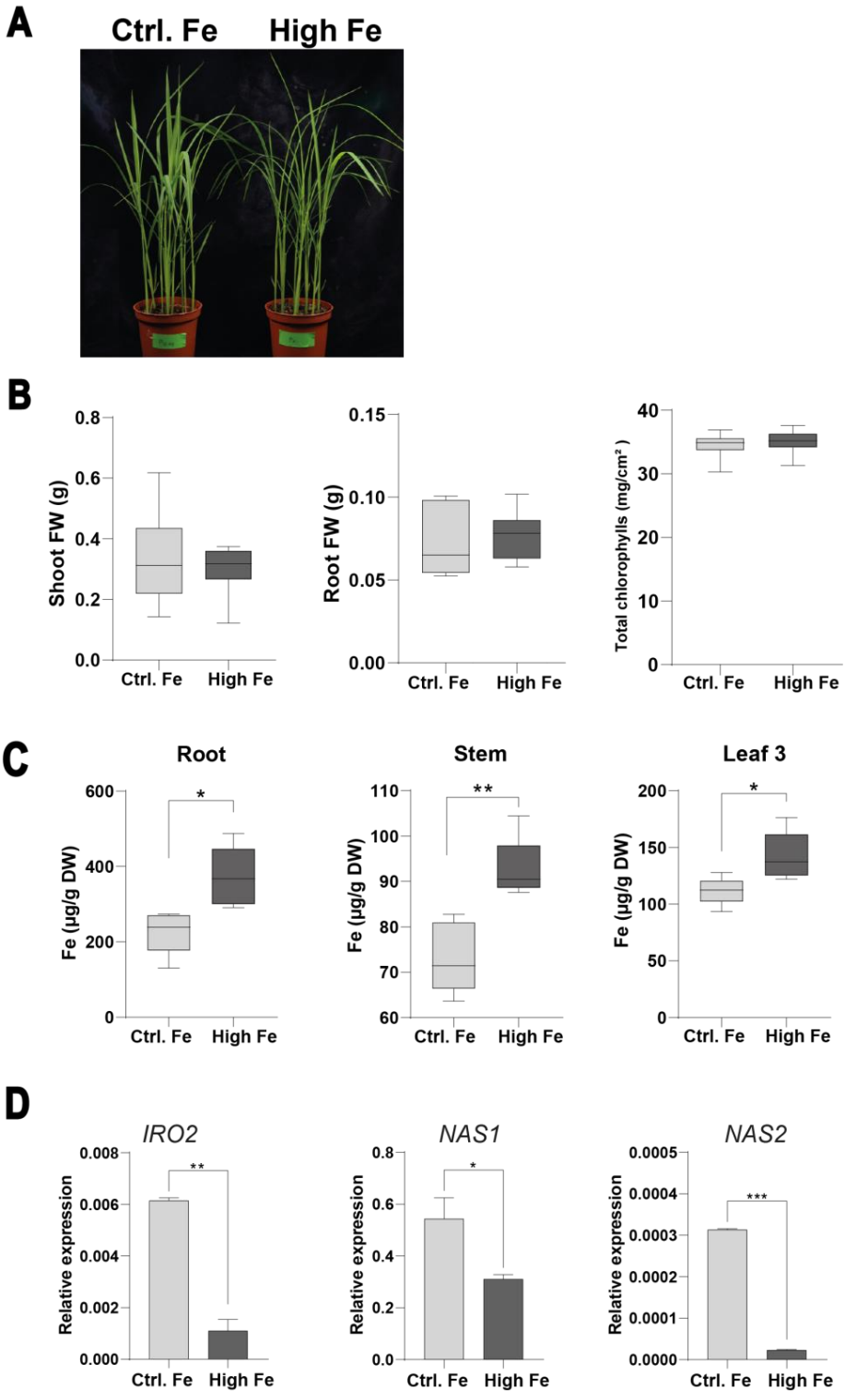
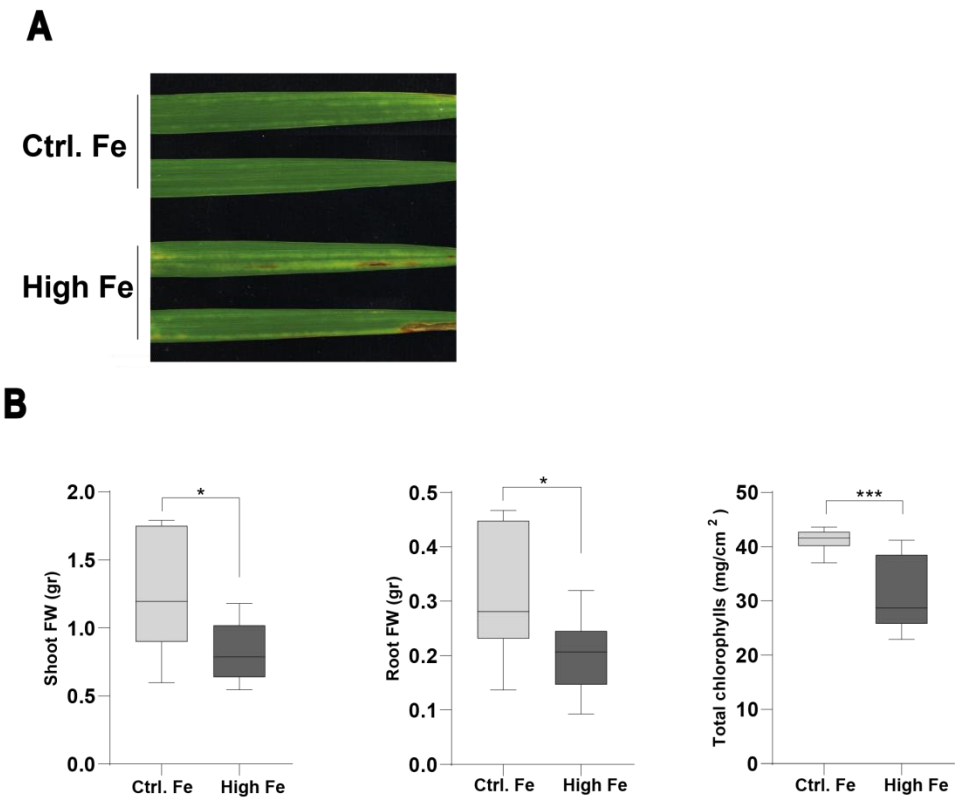
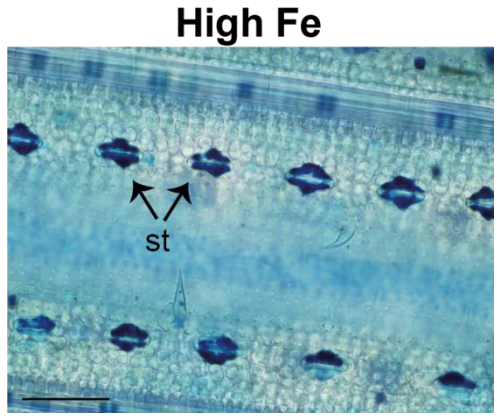
SUPPLEMENTAL FIGURES

Supplemental Figure S1. Phenotype of rice plants that have been grown under control Fe (0.05 mM Fe, control plants) or high Fe (1 mM Fe, High-Fe plants) supply. Phenotypical parameters were assayed at 5 days of Fe treatment. **(A)** Appearance of control and high-Fe plants. (**B)** Shoot and root fresh weight (FW) (left and middle panels). Chlorophyll content (right panel). Differences were not statistically significant. **(C)** Total Fe content estimated by the ferrozine colorimetric method in roots, stem and leaf treated as in (A). Data are mean ± SEM (n=10). Asterisks indicate statistical significant differences calculated by *t*-test (*, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively). **(D)** Expression of Fe deficiency responsive genes in roots of high-Fe and control plants after 5 days of Fe treatment. Transcript levels were determined by RT-qPCR analysis. The expression values were normalized to the rice *Ubiquitin1* gene. Three independent biological replicates (2 technical replicates each) were assayed. Data are mean ± SEM. Asterisks indicate statistical significant differences calculated by *t*-test (*, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively). Gene-specific primers are listed in **Supplemental Table S8**.

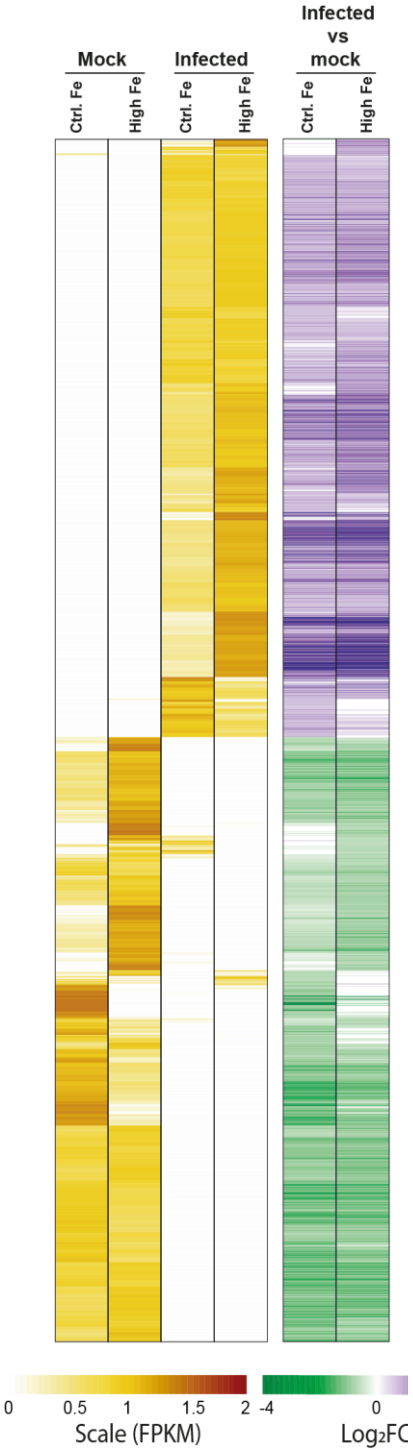

A
Ctrl. Fe
High Fe
B
Shoot FW (g)
0.8
0.6
0.4
0.2
0.0
Ctrl. Fe
High Fe
Root FW (g)
0.15
0.10
0.05
0.00
Ctrl. Fe
High Fe
Total chlorophylls (mg/cm²)
40
30
20
10
0
Ctrl. Fe
High Fe
C
Root
Fe (µg/g DW)
600
400
200
0
*
Ctrl. Fe
High Fe
Stem
Fe (µg/g DW)
110
100
90
80
70
60
**
Ctrl. Fe
High Fe
Leaf 3
Fe (µg/g DW)
200
150
100
50
0
*
Ctrl. Fe
High Fe
D
IRO2
Relative expression
0.008
0.006
0.004
0.002
0.000
**
Ctrl. Fe
High Fe
NAS1
Relative expression
0.8
0.6
0.4
0.2
0.0
*
Ctrl. Fe
High Fe
NAS2
Relative expression
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
***
Ctrl. Fe
High Fe



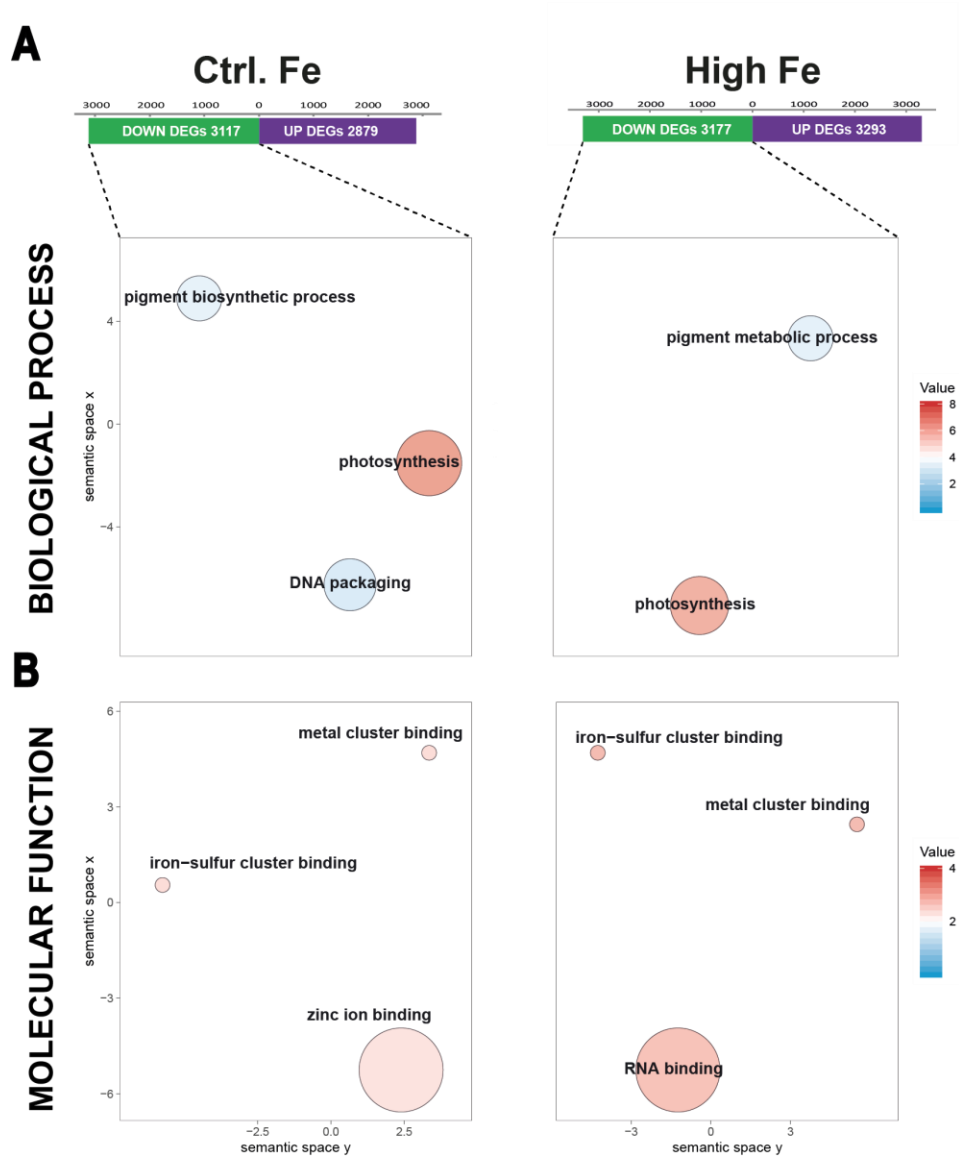
Supplemental Figure S2. Phenotype of rice plants that have been grown under control Fe (0.05 mM Fe, control plants) or high Fe (1 mM Fe) supply. Phenotypical parameters were assayed at 19 days of treatment with high Fe. **(A)** Appearance of control and high-Fe plants. **(B)** Shoot and root fresh weight (FW) (left and middle panels). Chlorophyll content (right panel). Data are mean ± SEM (n=10). Asterisks indicate statistical significant differences calculated by *t*-test (* and *** indicate $p < 0.05$ and 0.001, respectively).



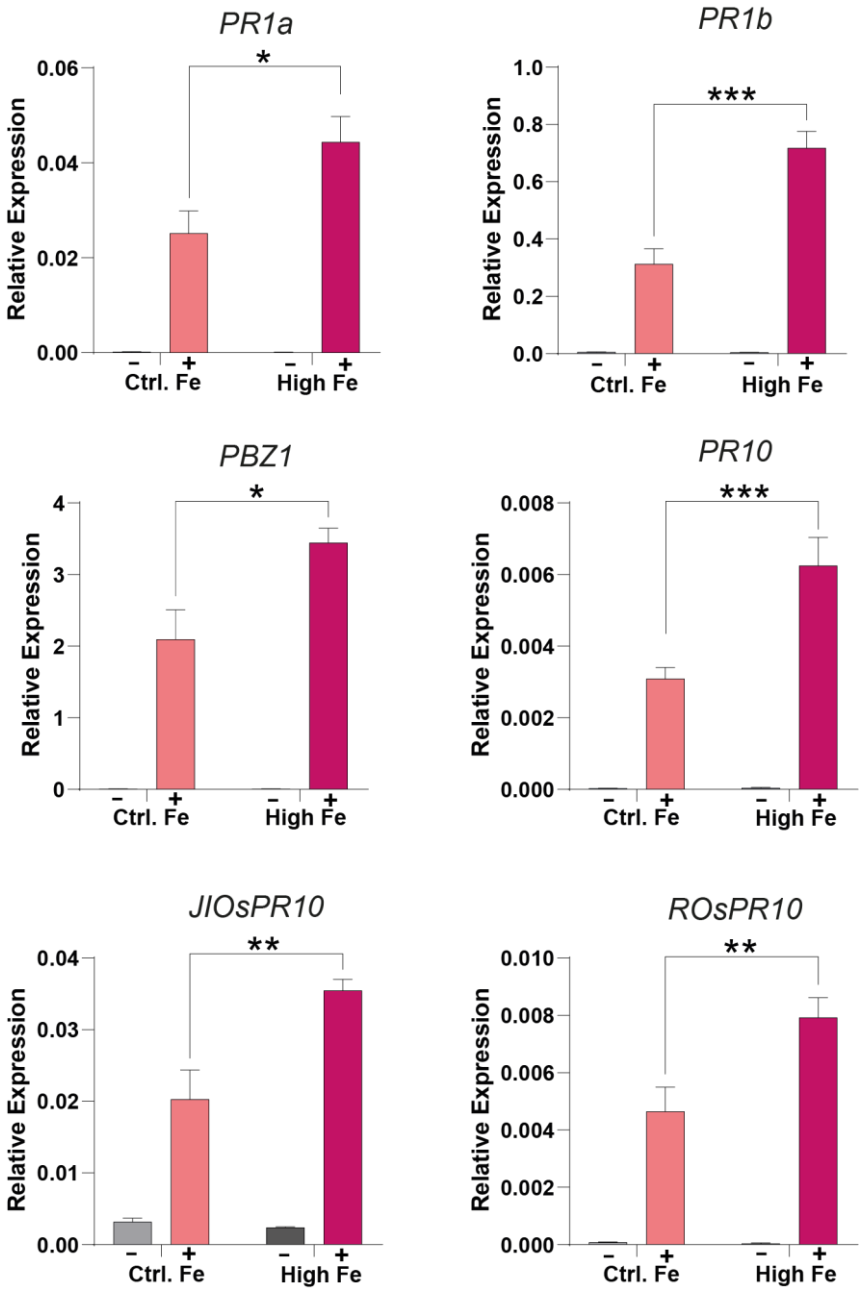
Supplemental Figure S3. Accumulation of iron (Ferric ions, Fe^{3+}) in leaves of control and high-Fe rice plants. The third leaf was stained Prussian Blue (Perls reagent (blue color)). Bar, 50 μm.



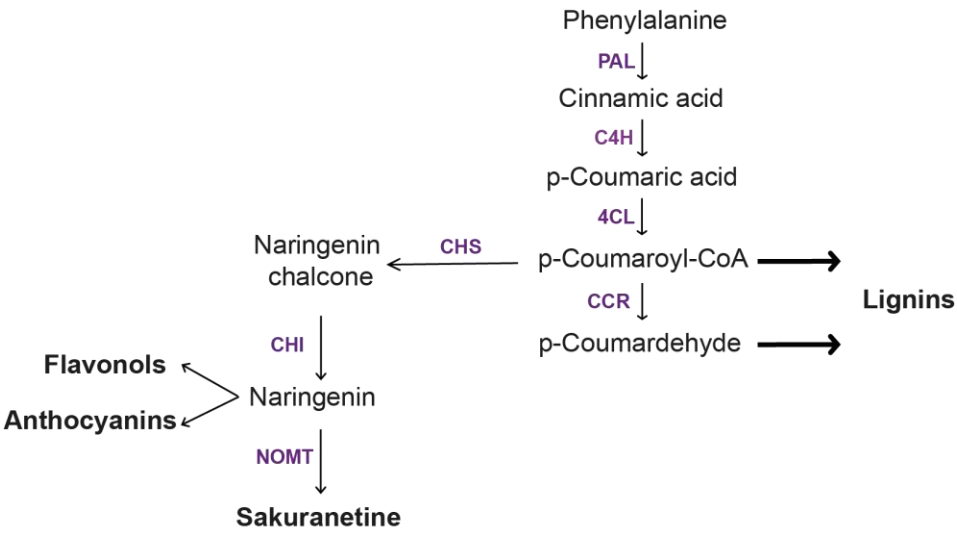
Supplemental Figure S4. Heat map depicting the expression of genes (row scale FPKM) whose expression is regulated by *M. oryzae* infection in control and high-Fe rice plants. Right panel, expression level (row scaled FPKM) is represented from pale yellow (less expressed) to brown (more expressed). Left panel, up-regulated genes (Log_2 fold change (FC) $\geq$ + 0.5; purple) and down-regulated genes (Log_2 FC $\geq$ - 0.5; green) DEGs. Data represented correspond to the mean of two biological replicates, each biological replicate consisting in a pool of 5 leaves from individual plants.



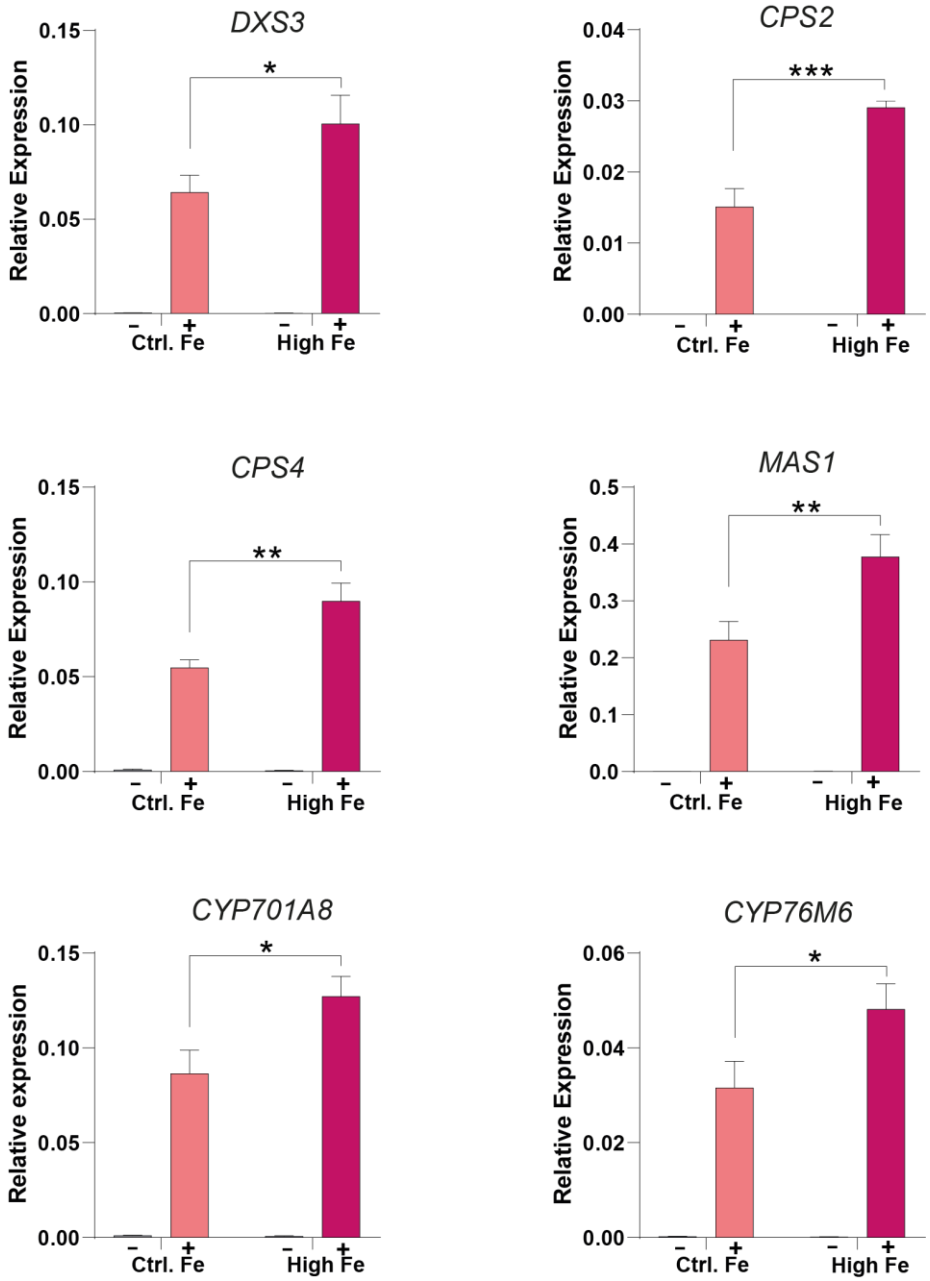
Supplemental Figure S5. GO enrichment analyses of genes down-regulated by *M. oryzae* infection in control and high-Fe plants (48 hpi) in the categories of Biological Processes **(A)** and Molecular Function **(B)**. GO terms were visualized using REVIGO (https://revigo.irb.hr/) after reducing redundancy and clustering of similar GO terms in the *O. sativa* database. GO terms are represented by circles and are clustered according to semantic similarities (more general terms are represented by larger size circles, and adjoining circles are most closely related). Circle size is proportional to the frequency of the GO term, whereas color indicates the enrichment derived from the AgriGO analysis (red higher, blue lower). Full data sets of DEGs and lists of complete GO terms are presented in **Supplemental Table S2** and **S3,** respectively).



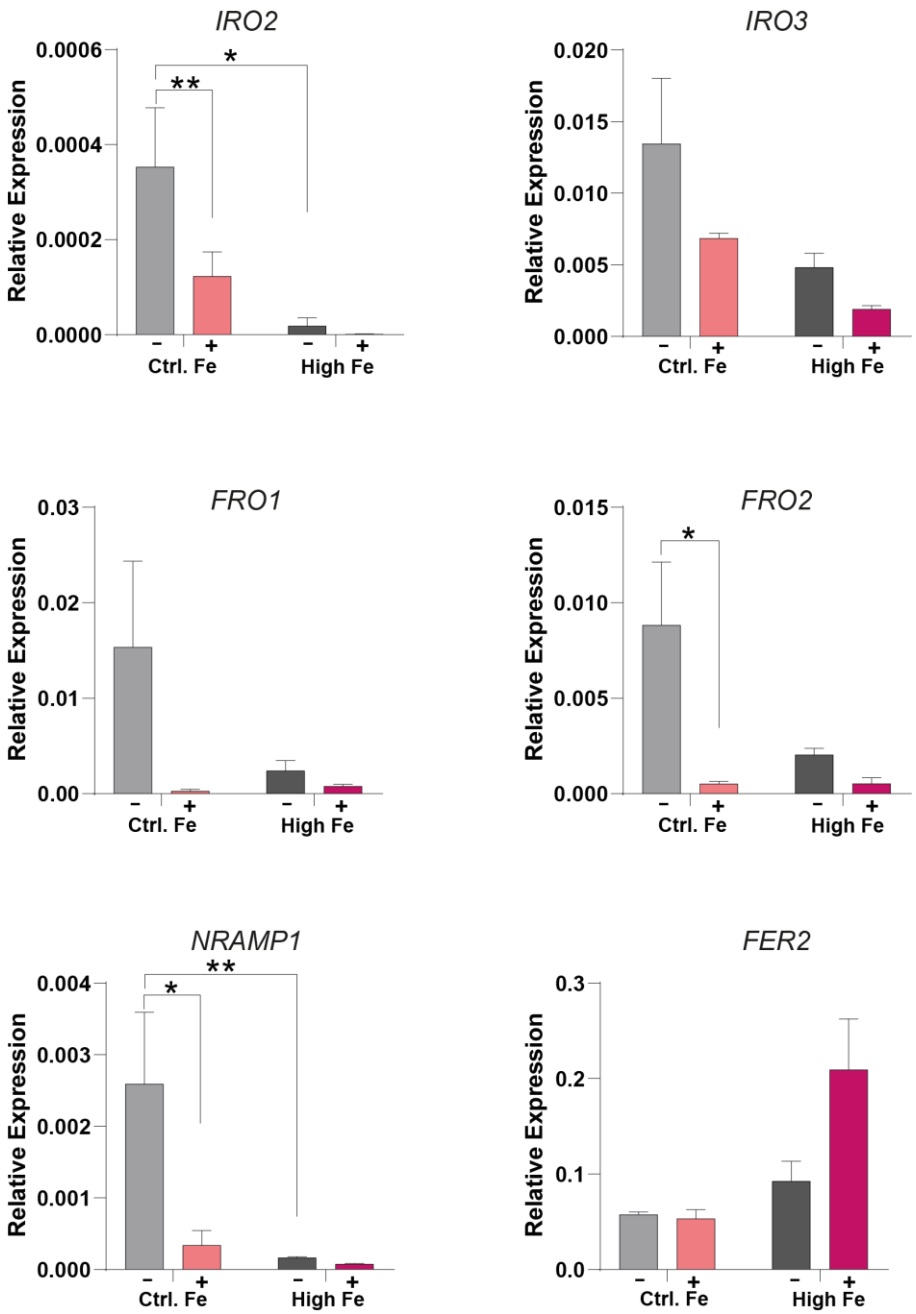
Supplemental Figure S6. Expression of *PR* genes in leaves of control and high-Fe plants (-, mock-inoculated; +, *M. oryzae*-inoculated). Transcript levels were determined by RT-qPCR analysis at 48 hpi. Expression values were normalized to the rice *Ubiquitin1* gene. Three independent experiments (2 technical replicates each) were carried out with similar results. Data are mean ± SEM (n=3). Asterisks indicate statistical significant differences calculated by two-way ANOVA (*, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively). Gene-specific primers are listed in **Supplemental Table S8.**



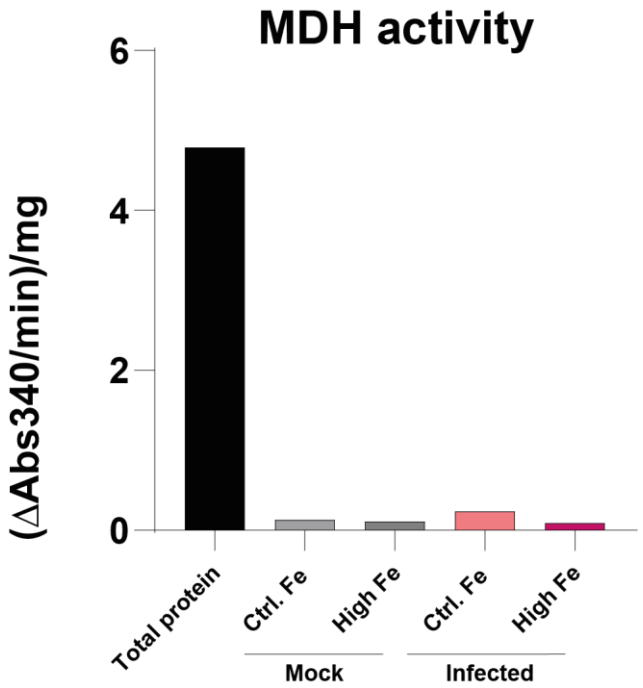
Supplemental Figure S7. Phenylpropanoid biosynthesis pathway. **PAL**, phenylalanine ammonia lyase; **C4H**, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; **CCR**, cinnamoyl-CoA reductase; **CHS**, chalcone synthase; **CHI**, chalcone isomerase; **NOMT**, naringenin 7-O-methyltransferase.



Supplemental Figure S8. Expression of diterpene phytoalexin biosynthetic genes in leaves of control and high-Fe plants (-, mock-inoculated; +, *M. oryzae*- inoculated). Transcript levels were determined by RT-qPCR analysis at 48 hpi. The expression values were normalized to the rice *Ubiquitin1* gene. Three independent biological replicates (2 technical replicates each) were assayed. Data are mean ± SEM (n=3). Asterisks indicate statistical significant differences calculated by two-way ANOVA (*, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively). Gene-specific primers are listed in **Supplemental Table S8**.



Supplemental Figure S9. Expression of genes involved in Fe homeostasis in leaves control and high-Fe plants (-, mock-inoculated; +, *M. oryzae*- inoculated). Transcript levels were determined by RT-qPCR analysis at 48 hpi. The expression values were normalized to the rice *Ubiquitin1* gene. Three independent biological replicates (2 technical replicates each) were assayed. Data are mean ± SEM (n=3). Asterisks indicate statistical significant differences calculated by two-way ANOVA (*, **, and *** indicate $p < 0.05$, 0.01, and 0.001, respectively). Gene-specific primers are listed in **Supplemental Table S8**.



Supplemental Figure S10. Malate dehydrogenase assay in total protein extracts or apoplast washing fluid obtained from control and high-Fe plants (mock-inoculated and *M. oryzae*-inoculated). Five biological replicates for each condition (with 10 plants per condition were analyzed. Representative results are presented.

Chapter III

Topical application of miRNAs targeting the mitogen-activated protein kinase *PMK1* gene of *Magnaporthe oryzae* enhances resistance to the rice blast fungus

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Manuscript in preparation

ABSTRACT

Small RNAs are short non-coding RNAs that guide gene silencing in most eukaryotes. There are two main classes of small RNAs in eukaryotes, small interfering RNAs (siRNAs) and microRNAs (miRNAs). While siRNAs derive from the processing of long double-stranded RNA molecules (dsRNAs), miRNAs are produced from single-stranded RNA precursors with unique stem-loop structures. Recent findings demonstrated that topical application of dsRNAs and siRNAs is effective for the control of fungal diseases in plants. This process relies on the capability of siRNAs to silence genes relevant for fungal development and/or pathogenicity, a phenomenon known as cross-kingdom RNA interference (RNAi). In this work, we demonstrate that tailor-made miRNAs, when topically applied onto rice leaves, are capable of preventing infection by the fungal pathogen *Magnaporthe oryzae*, the causal agent of the rice blast disease. Specifically, miRNA molecules targeting *MoPMK1* (Mitogen-activated protein kinase 1) involved in appressorium formation and cell-to-cell movement of *M. oryzae* was found to reduce leaf blast severity. This antifungal effect is observed by using either mature miRNAs species (e.g. miRNA duplexes) or miRNA precursor molecules. We also show that miRNA molecules can induce systemic silencing of *MoPMK1* and blast resistance at sites distant from the infected site. As *M. oryzae* infects multiple grasses and cereals, miRNA-based strategies can be useful in protecting plant species of agronomical interest from blast infection.

INTRODUCTION

RNA interference, or RNAi, is a conserved mechanism of gene silencing in most eukaryotes (Baulcombe 2004; Vaucheret 2006). RNAi is based on the regulatory function of short noncoding RNAs, or small RNAs of approximately 21-24 nucleotides in length, that guide silencing of target genes (Hung and Slotkin 2021; Kamthan *et al.* 2015; Singh *et al.* 2018). In plants, small RNAs can be categorized into two major classes, including small interfering RNAs (siRNAs) and microRNAs (miRNAs). Both miRNAs and siRNAs bind to complementary sites in their target mRNAs, and negatively regulate target gene expression.

MiRNAs and siRNAs differ among them by their distinct biogenesis pathways. Plant miRNAs are transcribed by RNA polymerase II from *MIR* genes as a long primary transcript that adopts a unique stem-loop structure that is sequentially processed by a RNase III DICER-like (typically DCL1) to produce double-stranded miRNA duplexes (miRNA-5p/miRNA-3p) (Arikit *et al.* 2013; Axtell 2013; Narjala *et al.* 2020; Rogers and Chen 2013). The two strands of the duplex are methylated at the 3' end and the functional strand of the duplex associates with Argonaute 1 (AGO1) to form a RNA-induced silencing complex (RISC) (Vaucheret 2008). The RISC facilitates sequence-specific cleavage or translation repression of the target transcripts (Brodersen *et al.* 2008; Llave *et al.* 2002). It is well recognized that miRNAs are key regulators of gene expression in diverse aspects of plant growth and development, as well in adaptive responses to abiotic and biotic stresses, including plant defense responses against pathogen infection (Alptekin *et al.* 2017; Chiou *et al.* 2006; Hackenberg *et al.* 2013; Koroban *et al.* 2016; Liang and Yu 2010; Pant *et al.* 2008; Zhai *et al.* 2011). Whereas miRNAs are produced from single-stranded RNA precursors with stem-loop precursors with an imperfect intramolecular hairpin structure, siRNAs derive from long double-stranded RNAs (dsRNAs) with perfect base-pair complementarity that are produced by RNA-dependent RNA polymerases (RDRs). Both siRNAs and miRNAs can move locally and systemically in plants through

plasmodesmata and phloem, respectively (Marín-González and Suárez-López 2012).

Increasing evidence support that small RNAs, siRNAs and miRNAs, play a critical role in regulating plant-pathogen interactions (Bundó *et al.* 2020; Song *et al.* 2021; Wang and Dean 2019; Weiberg *et al.* 2013, 2014, 2015). Regulation by small RNAs is not restricted to the individual organism in which they are produced due to the fact that small RNAs can move between host and pathogens, silencing genes in the interacting organism (Hua *et al.* 2018; Weiberg *et al.* 2015; Zotti *et al.* 2018). This phenomenon, known as cross-kingdom RNAi, has been described in several plant-pathogen interactions (Knip *et al.* 2014; Wang *et al.* 2016). Most of these studies have focused on the movement of siRNAs between pathogens and plants, and less is known about the movement of miRNAs in plant/pathogen interactions.

Regarding small RNA-mediated trans-kingdom gene regulation, it has long been demonstrated that transgenic expression in plants of siRNA-generating dsRNAs constructs targeting fungal transcripts protects the plant from an invading fungal pathogen by silencing the target gene in the pathogen, a phenomenon referred to as host-induced gene silencing (HIGS). For instance, movement of siRNAs occurs in barley and wheat plants infected with *Blumeria graminis*, barley plants infected with *Fusarium graminearum*, Arabidopsis and tomato plants infected with *Botrytis cinerea*, or cotton plants infected with *Verticillium dahliae* (Chen *et al.* 2016; Guo *et al.* 2014; Koch *et al.* 2013; Nowara *et al.* 2010; Panwar *et al.* 2018; Song and Thomma 2018; Wang and Dean 2019; Zhang *et al.* 2016a). However, although HIGS seems to be highly efficient for crop protection, its applicability is limited by the actual legislation and social concerns on genetically modified organisms (GMOs). The lack of efficient transformation protocols might also hinder the use of this strategy for disease control in crop species.

Cross-kingdom RNAi is bidirectional during plant-pathogen interactions (Wang *et al.* 2016). In this way, both plants and pathogens,

produce small RNAs that can target genes in their counterparts for gene silencing, and this cross-kingdom communication can determine the outcome of infection (Wang *et al.* 2016). Studies in the interaction of Arabidopsis and tomato plants with *B. cinerea* illustrates bidirectional cross-kingdom RNAi and sRNA trafficking between plants and fungi. Here fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways, thus, demonstrating that pathogens have evolved RNAi mechanisms to counteract plant immune responses (Weiberg *et al.* 2013). On the other hand, expressing small RNAs that target *B. cinerea DCL* genes (*Bc-DCL*) in transgenic Arabidopsis and tomato plants silences *Bc-DCL* genes and attenuates fungal pathogenicity (Wang *et al.* 2016). Recent studies have revealed extracellular vesicles (EVs) as vehicles of small RNA exchange in cross-species and cross-kingdom RNAi (Cai *et al.* 2018; He *et al.* 2021; Wang and Dean 2019).

Not only siRNAs, but also miRNAs can move from plants to pathogens to suppress expression of fungal genes (Cai *et al.* 2018; Zhang *et al.* 2016b). For instance, cotton plants export miR166 and miR159 to *V. dahliae* for silencing fungal virulence genes. This study provided the first evidence of silencing of fungal genes by an endogenous host miRNA. Later on, it was reported that wheat miRNA1023 targeting alpha/beta hydrolase from *F. graminearum* suppresses fungal invasion (Jiao and Peng 2018). In other studies, miRNAs from the parasitic plant *Cuscuta campestris* were found to silence Arabidopsis genes during parasitism (Shahid *et al.* 2018). Altogether, this piece of information supports that cross-kingdom RNAi is a conserved mechanism with important implications in plant immunity and pathogen virulence.

Previous work also indicated that fungal pathogens can take up external small RNAs, and dsRNAs which are then processed into siRNAs, and induce the silencing of pathogen genes with complementary sequences (Koch *et al.* 2016a; Wang *et al.* 2016). These discoveries led to the development of a non-GMO alternative to HIGS, known as Spray-Induced Gene Silencing (SIGS), which is based in the exogenous application of dsRNAs or siRNAs to down-regulate the expression of

endogenous fungal genes (Dalakouras *et al.* 2019; Koch *et al.* 2016; Taning *et al.* 2020; Wang *et al.* 2016, 2017; Wytinck *et al.* 2020). External application of dsRNAs and siRNAs was found to reduce disease symptoms caused by *B. cinerea* infection in fruits (tomato, strawberry, grape), flower petals (rose) and vegetables (onion epidermis, lettuce) (Wang *et al.* 2016). Importantly, these findings demonstrated that exogenous application of siRNAs (or dsRNA precursor molecules) can be used for protection of crop species against pathogen infection. Recently, it was described that SIGS is dependent on the efficiency of dsRNA uptake by the pathogen (Qiao *et al.* 2021). As long dsRNAs generate a diverse set of siRNAs, a drawback of using long dsRNAs (or siRNAs derived from dsRNAs) is a possible lack of specificity in silencing fungal genes. At present, miRNAs have not been used in SIGS technology. The use of miRNAs could overcome nonspecificity problems.

Rice is the staple food for more than half of the world's population. One of the major factors currently limiting rice production is the rice blast disease caused by the fungal pathogen *Magnaporthe oryzae* (Fernandez and Orth 2018; Wilson and Talbot 2009). *M. oryzae* is a hemibiotroph pathogen, which establishes a biotrophic interaction with the host at the early infection stages, followed by a necrotrophic phase. In addition to rice, *M. oryzae* has the capacity to infect other grass species, including major cereals such as wheat, barley, oats, and finger millet (Langner *et al.* 2018). At present, the rice and wheat blast diseases represent an important threat to global food security.

In this work, we report that topical application of *in vitro* and *in vivo* synthesized miRNA duplex or miRNA precursor, designed to silence a fungal gene involved in pathogenicity is effective for protection of rice plants against *M. oryzae* infection. Tailor-made miRNAs targeting specific *M. oryzae* genes represent a useful tool to prevent diseases caused by the blast fungus.

RESULTS

Selection of target genes for miRNA-based control of *M. oryzae* infection

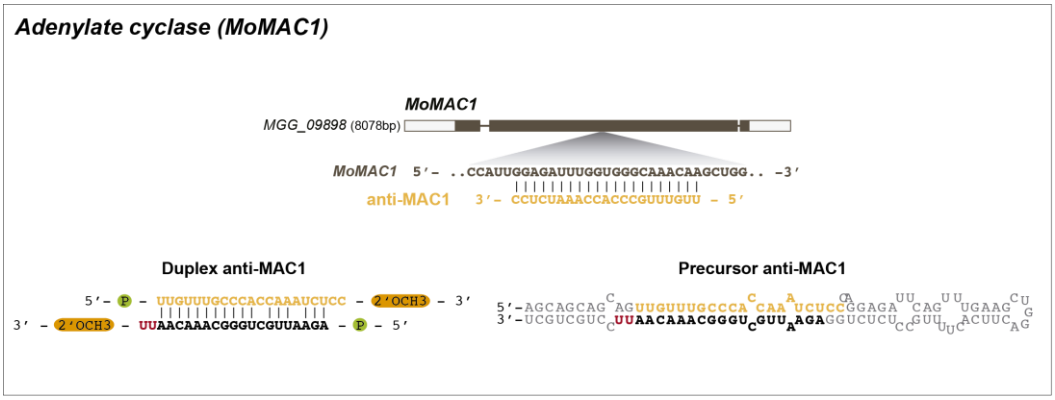
Two genes that are known to be involved in *M. oryzae* virulence and/or development were selected as potential targets for miRNA-based gene silencing. They were: *MoMAC1* and *MoPMK1*, encoding Adenylate Cyclase 1 MAC1 and the MAP (Mitogen-activated protein kinase) PMK1, respectively. The *MoMAC1* (MGG_09898) and *MoPMK1* (MGG_09565) coding sequences were retrieved from the *M. oryzae* database at the *Ensembl Fungi* website (https://fungi.ensembl.org/Magnaporthe_oryzae/Info/Index).

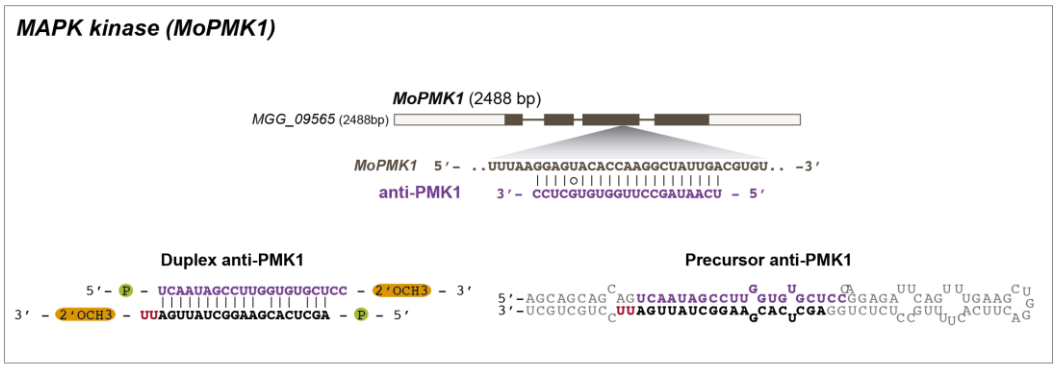
In *M. oryzae*, cAMP signaling is involved in surface recognition and pathogenesis (**Supplemental Figure S1**) (Choi and Dean 1997). *MoMAC1* encodes a membrane-bound enzyme that catalyzes the production of cAMP from ATP (Choi and Dean 1997; Zhou *et al.* 2012). MAC1 has a high degree of identity with other adenylate cyclase genes from several filamentous fungi as well as yeasts. *MoMAC1* deletion impairs appressorium formation and host infection (Zhou *et al.* 2012). The second *M. oryzae* gene selected for this study, *MoPMK1*, is also essential for appressorium formation and pathogenicity and it is conserved in many plant pathogenic fungi (Xu and Hamer 1996). Specifically, *MoPMK1* functions as a master regulator of appressorium development that acts downstream of the cAMP signal (**Supplemental Figure S1**) (Sakulkoo *et al.* 2018). Major components of leaf waxes are recognized by the rice blast fungus as chemical cues for the activation of the *Pmk1* pathway for appressorium formation (Liu *et al.* 2011). Then, *pmk1* mutants of *M. oryzae* fail to form appressoria and are not able to grow invasively in rice plants (Zhao *et al.* 2005; Zhou *et al.* 2014). More recently, *MoPMK1* was reported to participate in cell-to-cell movement of the fungus *via* plasmodesmata by controlling hyphal constriction that

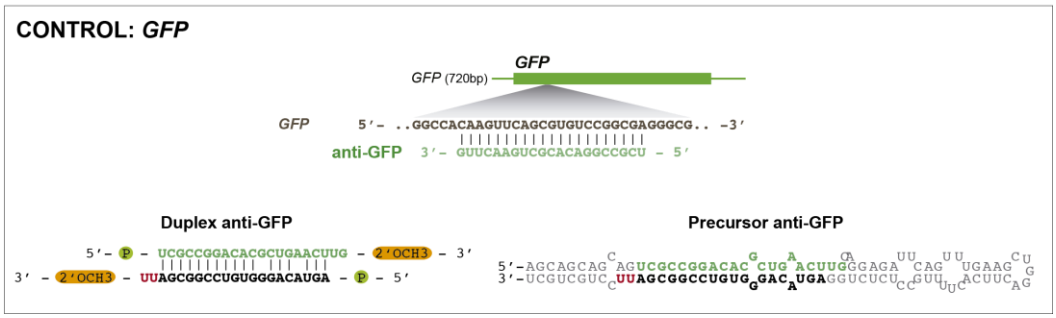
enables host tissue colonization and blast disease (Sakulkoo *et al.* 2018). Additionally, *MoPMK1* regulates the expression of secreted fungal effector proteins that interfere with the host immune response by preventing reactive oxygen species (ROS) generation and callose deposition at plasmodesmata (Sakulkoo *et al.* 2018). The involvement of *MoPMK1* in various stages at the biotrophic stage of the infection processes makes this gene a good candidate to assess the effectiveness of miRNA-based strategies for the control of *M. oryzae* infection.

The nucleotide sequences of small RNAs assayed in this work for transcriptional inhibition of *MoPMK1* and *MoMAC1* expression, and the recognition site in fungal target genes, are presented in **Figure 1** (upper panels for each gene). The full nucleotide sequence, and miRNA recognition sites, for each fungal gene can be seen in **Supplemental Figure S2**. As control, we designed a small RNA that targets Green Fluorescent Protein (GFP) to be assayed either as a control RNA in experiments of topical application of small RNAs, as well as in infection experiments with a *gfp*-expressing *M. oryzae* strain. Control anti-GFP small RNA was designed avoiding off-target effects in rice or *M. oryzae*. Thus, application of anti-GFP small RNA did not affect *M. oryzae* growth and infectivity.

Figure 1. Design of small RNA and precursor sequences. Schematic representation of the recognition site of small RNAs in the corresponding fungal target gene, *MoMAC1* (Adenylate Cyclase 1) and *MoPMK1* (Mitogen-activated protein kinase). Duplexes formed by chemically-synthesized small RNAs, as well as the structure of miRNA precursors in the *MIR528* backbone are shown. The same information is presented for small RNAs designed for silencing the *Green Fluorescent Protein* (*GFP*) gene.







Different strategies have been assayed in this work to evaluate the feasibility of using small RNA-based strategies for silencing selected fungal genes (**Figure 2**) (*MoMAC1* and *MoPMK1*), which included the use of small RNAs forming a possible miRNA-5p/miRNA-3p duplex or miRNA precursor designed to give rise a miRNA (**Figure 1,** left and right panels for each gene, respectively). For production of the various RNA molecules, we used *in vitro* and *in vivo* synthesis approaches, namely (i)

chemical synthesis for the preparation of small RNA duplexes (like miRNA-5p/miRNA-3p duplexes). As chemically synthesized miRNA duplexes are not the products of DCL processing of a precursor molecule, in this work we refer to these small RNA molecules as "miRNAs"; (ii) *in planta*-synthesized precursor sequences that can potentially be processed to produce mature miRNA sequences. It involved agroinfiltration of precursor miRNAs in *Nicotiana benthamiana* leaves and purification of the small RNA fraction from the agroinfiltrated leaves; and (iii) *in vitro* transcription of miRNA precursors in a cell-free system.

Synthesis of mature and precursor miRNAs

A major challenge in the development of an efficient miRNA-based approach for silencing fungal genes is the design of the miRNA sequence. Based on criteria established for miRNA biogenesis and target gene recognition (Axtell and Meyers 2018), the following requisites were considered to identify the most appropriated nucleotide sequences of "miRNAs". First, the two small RNAs of a duplex were 21 nucleotides (nt) in length, and formed a duplex with a 2-nucleotide overhang at their 3' ends, a signature of DCL activity **(Figure 1**, lower, left panel for each gene**)**. Small RNA should contain a maximum of 4 mismatches in the duplex. Second, the small RNA representing the functional "miRNA" had perfect complementarity with the recognition site at the target gene from nucleotides 1 to 14 (starting by the 5' small RNA sequence). **(Figure 1**, upper panels for each gene). The most commonly observed 5′ nucleotide of functional miRNAs in plants is uracil, and results previously reported on miRNAs that are exported from cotton plants to the fungal pathogen *V. dahliae* confirmed silencing of fungal genes (Zhang *et al.* 2016b). Accordingly, small RNAs designed in this study to silence *M. oryzae* genes contained uracil at its 5' end. No mismatches between the 3' end of the small RNA and the target sequence were allowed, the recognition site of the small RNA being located in the coding sequence of the target gene. Similar to *in vivo* synthesized "miRNAs", the *in vitro*-synthesized

"miRNAs" contained a 5' phosphate group, a 3' methyl group, and formed a duplex with 2-nt 3' overhangs **(Figure 1**, lower, left panel for each gene**)**. Following these criteria, candidate "miRNA" sequences for silencing *MoMAC1* and *MoPMK1*, as well as *GFP*, were designed **(Figure 1)**.

We also searched for recognition sites with minimal off-target effects. The pSRNAtarget tool (https://plantgrn.noble.org/psRNATarget/) was used to identify possible off-targets for each small RNA of interest, both in rice and *M. oryzae*. Small RNAs on-target score and off-target scores are presented in **Supplemental Table S1**. The miRNA sequences assayed in this work were named as anti-MAC1, anti-PMK1 and anti-GFP **(Supplemental Table S1)**.

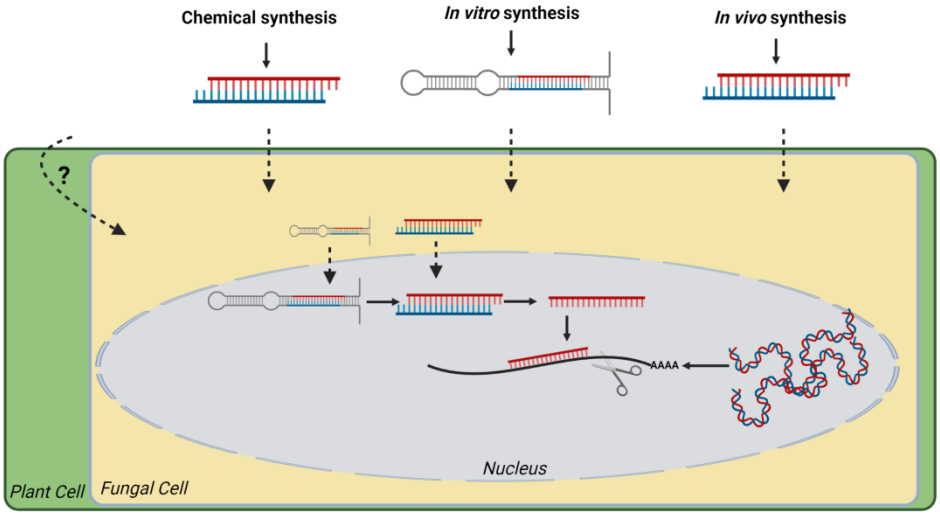


Figure 2. General overview of strategies used for RNA synthesis. (i) Chemical synthesis of small RNAs (duplexes), (ii) *in vitro* synthesis of miRNA precursors, and (iii) *in vivo* synthesis of miRNA duplexes.

In this study, we also evaluated the potential of applying miRNA precursors on rice leaves which, upon processing by the miRNA biogenesis machinery, could target fungal genes. Though it has been reported that biogenesis of certain miRNAs resembles that in plants and requires DICER RNases for precursor miRNA processing (Campo *et al.*

2016; Chen *et al.* 2014), the biogenesis pathway for miRNAs (or miRNA-like RNAs, milRNAs) is still poorly understood in most phytopathogenic fungal species. To obtain an expression system for the *in vitro* and *in vivo* synthesis of miRNA precursors, we took advantage of the simplified method to generate artificial miRNAs (amiRNAs) based on the naturally occurring miR528 precursor sequence of rice (http://weigelworld.org/). This sequence was used as a scaffold to produce a miRNA precursor harboring the small RNAs of interest. Upon processing, these precursors are expected to generate the desired miRNA sequences. We also took into consideration the possibility that an exogenously applied miRNA precursor could be incorporated by the plant cell to be then delivered to the fungal cells, if required. This strategy would offer new possibilities for silencing genes from phytopathogenic fungi by the exogenous application of miRNA precursors.

Agroinfiltration of *N. benthamiana* leaves was used for the *in vivo* production of miRNAs. In this system, the miRNA precursor sequences are expected to be synthesized and processed in the leaf tissue to produce mature miRNAs. The small miRNA sequence of interest (anti-MAC1, anti-PMK1, anti-GFP) was cloned into the pre-miR528 backbone (pNW55 vector; Warthmann *et al.* 2013; primers used are listed in **Supplemental Table 2).** Transient expression assays were carried out in leaves of 15-days-old *N. benthamiana* (**Figure 3A**, *Rdr6IR* line; Schwach *et al.* 2005 plants). 24 hours post inoculation (hpi) and 96 hpi after agroinfiltration, the tissues were analyzed by small RNA Northern blot analysis using oligonucleotide probes complementary to the corresponding small RNA (nucleotide sequences are shown in **Supplemental Table 2**). The expected small RNA sequences were produced from the amiRNA precursors at 24 hpi, its accumulation further increased at 96 hpi in the agroinfiltrated *N. benthamiana* leaves (**Figure 3B**). No signals were detected in control leaves agroinfiltrated with the empty vector (**Figure 3B**). Stem-loop RT-PCR validated that the expected mature miRNA sequences accumulated in agroinfiltrated *N.*

benthamiana leaves (**Figure 3C**). Thus, these results confirmed that the precursor structures are functional and produce the expected mature miRNAs. The small RNAs fraction was purified from agroinfiltrated leaves (96 hpi) and used for infection experiments (see below).

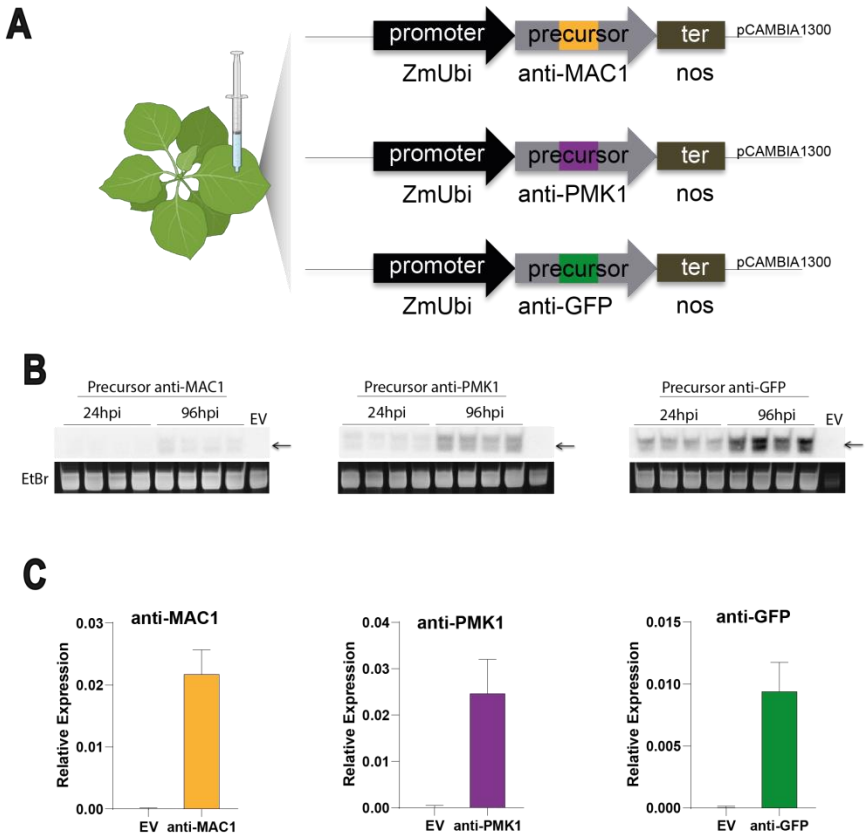


Figure 3. *In vivo* synthesis of miRNA duplexes by transient expression in *Nicotiana benthamiana* leaves**. (A)** Leaves from 15-days-old *N. benthamiana RDR6-IR* were agroinfiltrated with an expression vector harboring an artificial miRNAs designed for silencing *MoMAC1* (anti-MAC1), *MoPMK1* (anti-PMK1*)*, or GFP (anti-GFP) expression. Two times after agroinfiltration were analized (24h and 96h). (**B)** Northern blot analysis for detection of anti-PMK1, anti-MAC1 and anti-GFP accumulation in agroinfiltrated *N. benthamiana* leaves. The small RNA fraction obtained from total RNA (50 µg) was subjected to northern blot and probed with a P^{32}- labelled synthetic oligonucleotides complementary to the anti-PMK1, the anti-MAC1 or the anti-GFP small RNA sequences. RNA gels were stained with ethidium bromide (EtBr) for loading control (lower panel). Arrows indicate the small RNAs produced after processing of the corresponding miRNA precursors. Four biological replicates were assayed for each time (24 and 96 h). **(C)** Stem–loop RT–qPCR of *in vivo* synthetized miRNAs. Data from one representative experiment of two independent experiments is presented. Bars represent the mean ± SEM (four biological replicates, each replicate consisting in a pool of 5 leaves). Oligonucleotide sequences used as probes in B, and primers used for stem-loop RT-PCR are listed in **Supplemental Table S2**.

Moreover, a cell-free *in vitro* RNA synthesis was also used to obtain precursor miRNA sequences. For this, the precursor of interest was cloned into the pCR-Blunt vector and transcribed using the T7 RNA polymerase. *In vitro* transcription allowed to synthetize miRNA precursor molecules.

Local application of chemically-synthesized and in *vitro*-synthesized miRNAs targeting *MoPMK1* confers resistance to *M. oryzae* infection.

To assess the potential of "miRNAs" and miRNA precursors generated in this study for silencing the fungal genes, we conducted infection experiments with the rice blast fungus *M. oryzae*. MiRNAs, either the chemically-synthesized "miRNA" duplexes or the *in vitro*-synthesized miRNA precursors (**Figure 4**), as well as the *in vivo* synthesized miRNAs (**Figure 5**) were locally applied on rice leaves. The miRNA-treated leaves were then inoculated with *M. oryzae* spores as described in Material and Methods. Development of disease symptoms was monitored over time. Controls in these studies included the application of H_2O prior inoculation with fungal spores (no "miRNA" treatment). As a negative control we also used the anti-GFP "miRNA" (chemical, *in vitro* or *in vivo* synthetized treatment prior inoculation).

The application of chemically-synthesized anti-PMK1 prior inoculation with *M. oryzae* spores significantly reduced blast symptoms (**Figure 4A**). In contrast, the application of anti-MAC1 did not result in apparent reduction of disease symptoms (**Figure 4A**). As expected, no effect was observed in control leaves in which the anti-GFP or water was applied (**Figure 4A**). These results were confirmed by quantification of leaf areas showing blast lesions (**Figure 4A**, right panel). To confirm that the reduced infection upon treatment with anti-PMK1 was provoked by specific gene silencing, we examined the expression of fungal genes. This analysis revealed specific down-regulation of *MoPMK1* expression in

leaves that have been treated with anti-PMK1, but not in water-treated leaves (**Figure 4B**).

Similar results were obtained by topical application of *in vitro* synthetized miRNA precursors prior inoculation with fungal spores. Results are presented in **Figure 4C**. Again, an important reduction in blast symptoms was observed by visual inspection in leaves treated with the anti-PMK1 precursor, but not in leaves treated with the anti-MAC1 or anti-GFP precursors, or in control leaves (H_2O-treated) (**Figure 4C**), which was further confirmed by quantification of leaf areas showing blast lesions (**Figure 4C,** right panel). Similarly to what is observed with local application of chemically-synthesized anti-PMK1 (**Figure 4A**), a down-regulation of *MoPMK1* expression occurred in the rice leaves in which the anti-PMK1 precursor was applied (**Figure 4D**).

These results demonstrated that the application prior to infection of both *in vitro* synthesized "miRNAs" and miRNA precursors designed for *MoPMK1* silencing provokes down regulation of *MoPMK1* and confers protection to infection by the blast fungus *M. oryzae* in rice plants.

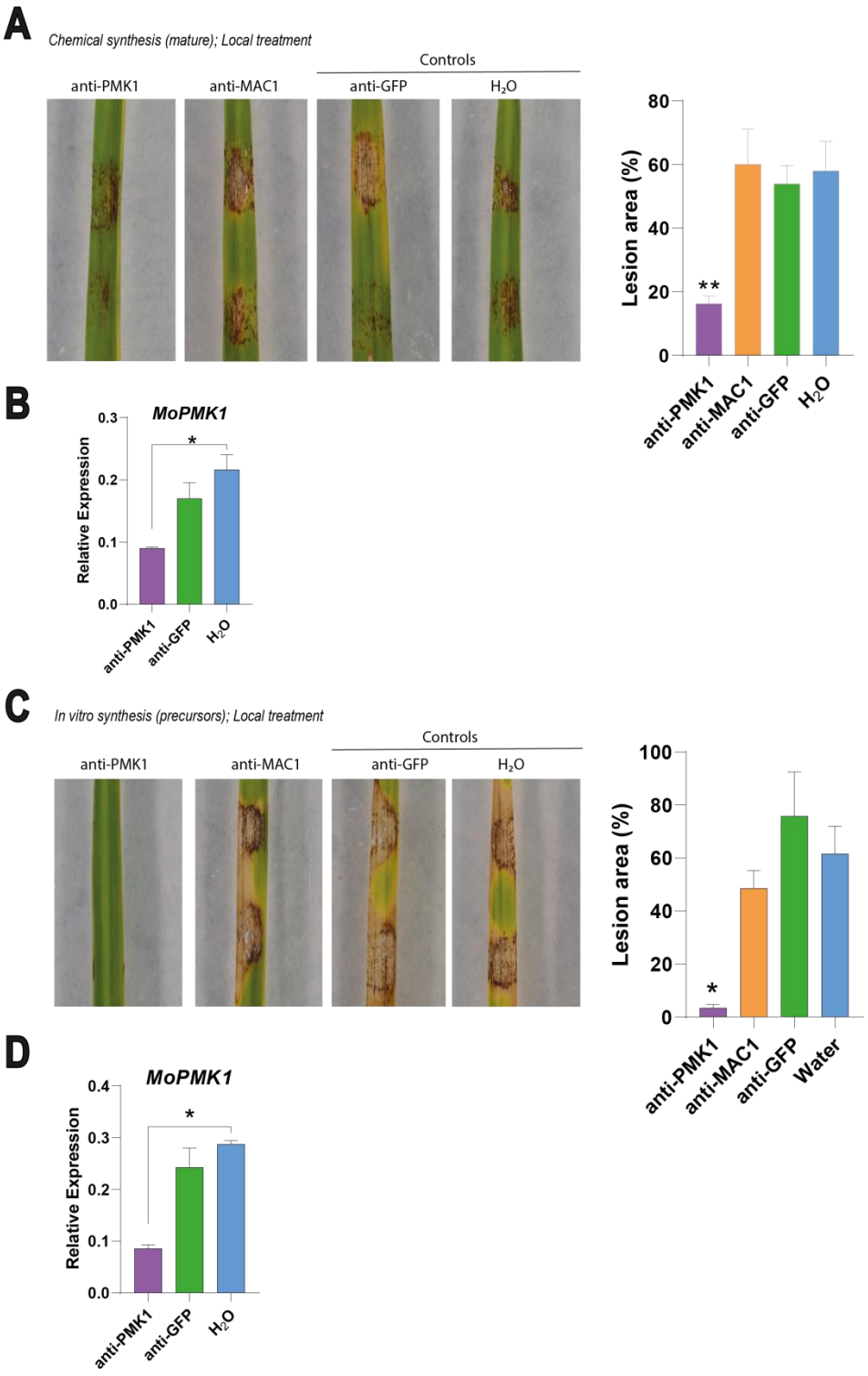

A
Chemical synthesis (mature); Local treatment
Controls
anti-PMK1
anti-MAC1
anti-GFP
H2O
Lesion area (%)
80
60
40
20
0
**
anti-PMK1
anti-MAC1
anti-GFP
H2O
B
MoPMK1
Relative Expression
0.3
0.2
0.1
0.0
*
anti-PMK1
anti-GFP
H2O
C
In vitro synthesis (precursors); Local treatment
Controls
anti-PMK1
anti-MAC1
anti-GFP
H2O
Lesion area (%)
100
80
60
40
20
0
*
anti-PMK1
anti-MAC1
anti-GFP
Water
D
MoPMK1
Relative Expression
0.4
0.3
0.2
0.1
0.0
*
anti-PMK1
anti-GFP
H2O

Figure 4. Resistance to *M. oryzae* infection in rice leaves treated with anti-PMK1 or anti-MAC1 "miRNA" **(A, B)** or precursor miRNAs **(C, D)**. The detached leaf assay was used in this study (Coca *et al.* 2004). Leaves of 3-week-old *O. sativa* var. Nipponbare were treated with the appropriate RNA solution (10 µl, at a concentration of 50 ng/µl). H_2O was applied in control leaves. The RNA-treated sites were then inoculated with *M. oryzae* spores (5 x 10^5 spores/ml). (**A, B)** Effect of treatment with *in vitro*-synthesized "miRNAs" (anti-PMK1, anti-MAC1, anti-GFP) on the development of blast lesions at 6 dpi **(A)**. Left panel, percentage of leaf area affected by blast lesions determined by image analysis (APS Assess 2.0) at 6 days after infection (4 biological replicates, and 4 inoculation sites each leaf). Transcript levels of *MoPMK1* **(B)** were determined by RT-qPCR in leaves that have been treated with either anti-PMK1, anti-GFP, or H_2O (3 biological replicates and 3 inoculation sites each leaf were assayed with similar results). (**C, D)** Effect of treatment with *in vitro*-synthesized miRNA precursors on *M. oryzae* infection. Left panel, percentage of leaf disease area (as in A). Transcript levels of *MoPMK1* (D) were determined by RT-qPCR in leaves that have been treated with miRNA precursors for either anti-PMK1 or anti-GFP (as in B). Data shown in B and D are the mean ± SEM (asterisks indicate statistically significant differences one way ANOVA *, $p \leq 0.05$; **, $p \leq 0.01$). Sequences of oligonucleotide primers are listed in **Supplemental Table S2**.

Local application of *in vivo*-synthetized anti-PMK1 confers resistance to *M. oryzae* infection

The *in vivo* synthesized miRNAs obtained from agroinfiltrated *N. benthamiana* leaves were used in infection experiments (**Figure 5**). The small RNA fraction (10 ul, 30 ng/µl) was topically applied on the rice leaves, which were then inoculated with *M. oryzae* spores. All the leaves treated with anti-PMK1-containing RNA showed reduced disease symptoms and lesion area compared with control leaves (e.g. leaves agroinfiltrated with the anti-GFP construct or with the empty vector, and H_2O-treated leaves). Although not statistically significant, a small reduction in diseased leaf area could be observed in leaves that have been treated with anti-MAC1 miRNAs (**Figure 5A**). Although the reduction of the infected areas upon application of *in vivo* synthesized anti-PMK1 (30% reduction) was comparable to that in leaves treated with chemically-synthesized anti-PMK1 "miRNA" (20% reduction) the *in vitro* synthetized anti-PMK1 precursors showed the higher fungal inhibition (90% reduction) (see **Figure 4**). Control leaves, treated with small RNAs obtained from *N. benthamiana* leaves transformed with the

anti-GFP precursor or the empty vector developed disease symptoms similar to those in H2O-treated leaves **(Figure 5A)**.

Expression analysis revealed specific down-regulation of *MoPMK1* and gene in leaves treated with anti-PMK1. The expression of target genes was unaltered in the three control conditions (empty vector, anti-GPF and water treatments) **(Figure 5B)**.

Together, these results indicate that topical application of *in vivo*-synthetized anti-PMK1 miRNAs protects rice plants from *M. oryzae* infection by specifically targeting *M. oryzae MoPMK1*.

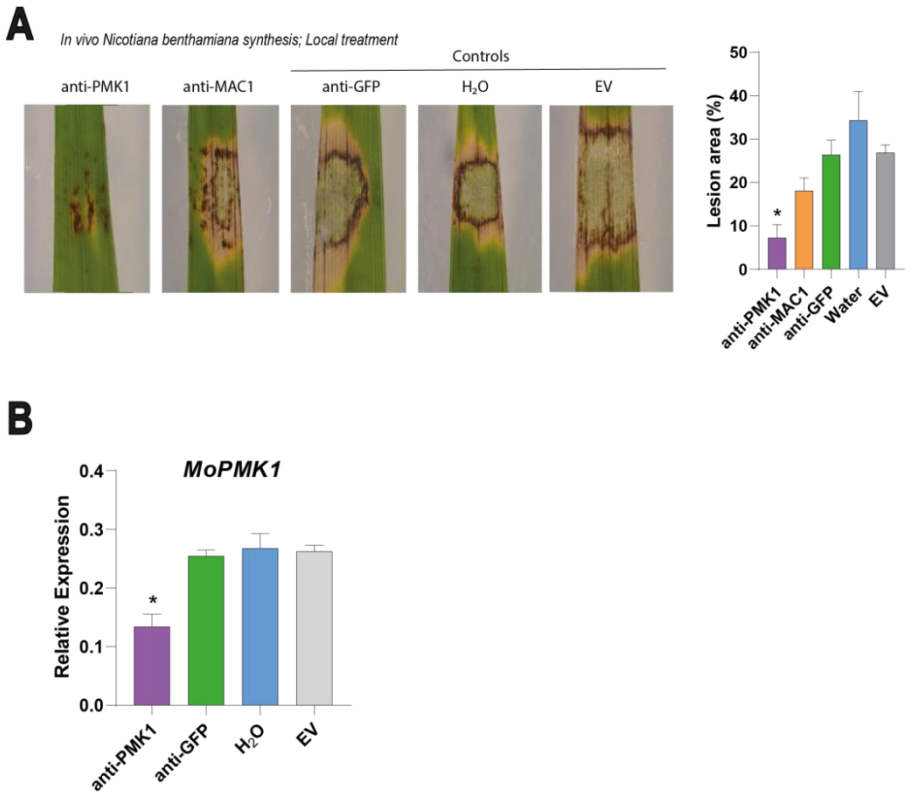


Figure 5. Resistance to *M. oryzae* infection in rice leaves treated with *in vivo*-synthesized miRNAs. The miRNAs were produced in *N. benthamiana* leaves. Disease symptoms at 6 days after inoculation with *M. oryzae* spores are shown. Left panel, percentage of leaf area affected by blast lesions at 6 dpi was determined by image analysis (APS Assess 2.0). **(B)** Transcript levels of *MoPMK1* were determined by RT-qPCR analysis of the miRNA-treated leaves at 6 dpi (3 biological replicates, and 3 inoculation sites each leaf). Data shown in histograms (A and B) are the mean ± SEM (asterisks indicate statistically significant differences calculated by one way ANOVA *, $p \leq 0.05$). Sequences of oligonucleotide primers are listed in **Supplemental Table S2**.

miRNA-mediated systemic silencing of *MoPMK1* reduces *M. oryzae* infection

Previous studies demonstrated that dsRNA can be absorbed by rice roots and are capable of silencing endogenous genes throughout the plant (Hunter *et al.* 2012; Li *et al.* 2015). This method overcomes the need of spraying entire plants which requires larger amounts of miRNAs. Later on, root soaking of dsRNAs was found to trigger plant and insect RNAi, thus, reducing damage caused by insect pests (Li *et al.* 2015). Moreover, the application of a dsRNA targeting the *lanosterol* gene from *F. graminearum* on detached barley leaves inhibited fungal growth not only in directly sprayed leaves but also in distal parts of the leaves (Koch *et al.* 2016b). On this basis, we investigated whether miRNAs could be absorbed by the rice leaves as a means for inhibition of *M. oryzae* infection in distal parts of the leaf. For this, the base of detached leaves was submerged in a solution containing the *in vitro* synthetized miRNA precursor (anti-PMK1 or anti-MAC1 precursor) (**Figure 6A**). Control leaves were submerged in anti-GFP precursor or water. Eighteen hours later, the rice leaves were inoculated with *M. oryzae* at a distance of approximately 5 cm from the submerged leaf segment. As shown in **Figure 6B** distal leaf areas of anti-PMK1-treated leaves developed substantially smaller lesions as compared with leaves treated with anti-MAC1 and anti-GFP precursor miRNAs and water-treated leaves, suggesting that anti-PMK1 RNA molecules are absorbed and translocated to distal parts of the leaf for silencing *MoPMK1*.

Collectively, results obtained in these studies indicated that application of “miRNAs”, or miRNA precursors, designed for silencing of *MoPMK1*, is accompanied by down-regulation of *MoPMK1* expression and protection against *M. oryzae* infection. The protective effect of anti-PMK1 is observed in both directly inoculated (local infection), as well as in non-treated (distal) leaf regions. Thus, miRNA-based strategies directed to silence *MoPMK1* are effective to arrest infection by the blast fungus in rice.

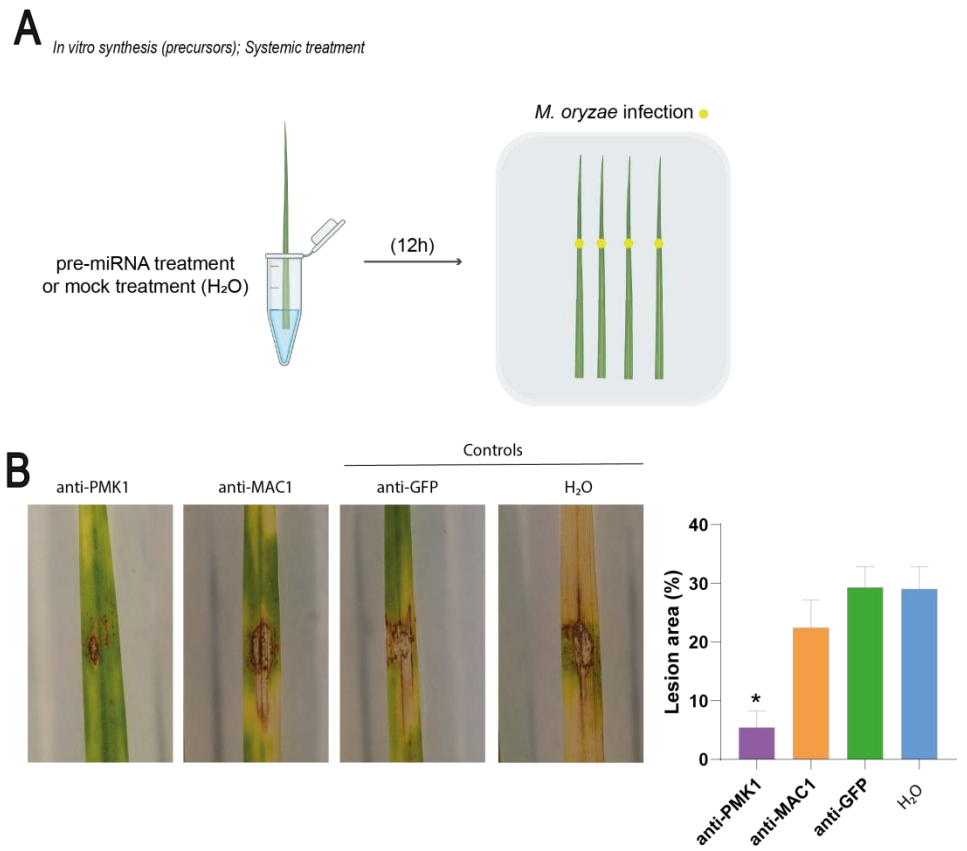


Figure 6. Treatment with anti-PMK1 confers systemic resistance to *M. oryzae* infection. **(A)** The base of rice leaves was submerged in a solution containing the miRNA precursor for the desired miRNA (anti-PMK1, anti-MAC1, or anti-GFP; 500 µl at a concentration of 50 ng/µl), and maintained at 28ºC under high humidity for 12 hours. Control leaves were submerged on sterile H_2O. Infection with *M. oryzae* spores (5 x 10^5 spores/ml) was carried out at distal parts (aprox. 5 cm) of the leaf base. **(B)** Detached infected leaves treated as in (A). The percentage of lesion areas was determined by image analysis (APS Assess 2.0) at 6 days after infection (right panel). Data are mean ± SEM (4 biological replicates). Asterisks indicate statistically significant differences calculated by one way ANOVA *, $p \leq 0.05$).

Topical application of anti-PMK1 compromises appressorium formation in rice leaves

The blast fungus *M. oryzae* uses appressoria to penetrate into the rice leaf tissue, and after penetration, the appressorium remnants remain on the leaf surface. Knowing that *MoPMK1* is a central regulator of appressorium formation and that pathogenicity is lost in *PMK1* mutants, we investigated whether treatment with anti-PMK1 has an effect on appressorium formation on the infected rice leaves. RNAs, either the chemically synthesized anti-PMK1 or the *in vitro* synthesized anti-PMK1 precursor were applied on the surface of rice leaves, which were then inoculated with *M. oryzae* spores (10 µl, x 50 ng/µl). Leaves were maintained under high humidity for 16 hours. Counting of appressoria present at each inoculated area revealed a significant reduction in the number of appressoria in areas where chemically-synthesized anti-PMK1 was applied compared with H_2O-treated leaves (**Figure 7**). Although not statistically significant, the *in vitro* synthetized anti-PMK1 precursor also caused a reduction on appressorium formation. However, under the same experimental conditions, the number of appressoria was not affected by treatment with anti-GFP or anti-GFP precursor molecules (**Figure 7**). Increasing the amount of anti-PMK1 RNA molecules (from 50 to 100 ng/µl) further reduced the number of appressoria found in the *M. oryzae*-infected areas (**Supplemental Figure S3**). This observation suggests that the application of anti-PMK1 RNAs, either "miRNA" or *in vitro*-synthesized anti-PMK precursor can cause dose-dependent reduction in appressorium formation in rice leaves.

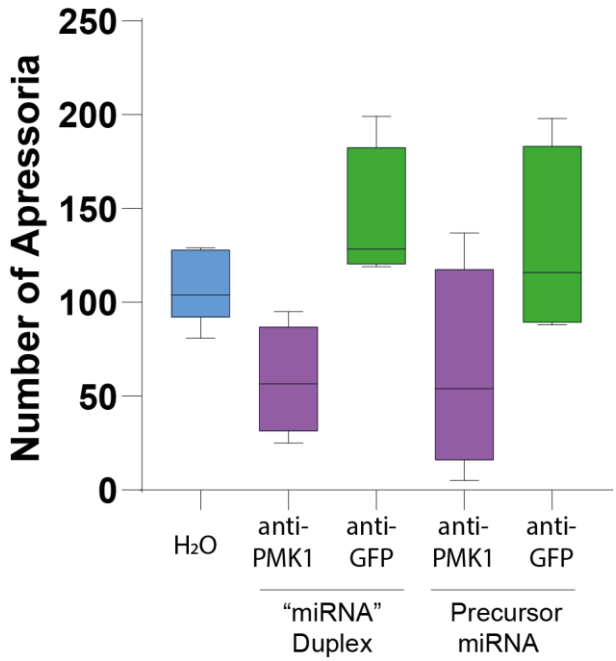


Figure 7. Inhibition of appressoria formation upon treatment with anti-PMK1. Rice leaves were treated with chemically-synthesized miRNAs duplexes (anti-PMK1 or control anti-GFP) or the corresponding *in vitro*-synthesized miRNA precursors (10 µl, 50 ng/µl). Control leaves were treated with sterile H_2O. The number of appressoria on the leaf surface were counted at 16 hours after fungal inoculation.

Treatment with anti-PMK1 inhibits *M. oryzae* infection in rice leaf sheaths

The rice leaf sheath is commonly used for live cell imaging of cytological changes occurring during *M. oryzae* infection by using *gfp*-expressing *M. oryzae* strains (Koga *et al.* 2004). In this work, the leaf sheath assay was used to further investigate the effect of treatment with anti-PMK1 miRNA on the *M. oryzae* infection process. For this, a solution containing *in vitro* synthesized anti-PMK1 (50 µl, 20 ng/µl), was applied to the inner epidermis of excised leaf sheaths together with a spore solution of a *gfp*-expressing *M. oryzae* strain (*GFP-PR9*) (Campos-

Soriano and San Segundo 2009). GFP fluorescence was monitored by confocal microscopy at 24 hpi (hours post-inoculation). The presence of infection structures beneath appressoria was evaluated in the infected sheaths.

In non-treated leaf sheaths (H_2O-treated + *M. oryzae* spores), GFP fluorescent *M. oryzae* hyphae were detected in the first invaded epidermal cell as well as in neighboring cells (**Figure 8,** upper panel). In sheath leaves that have been treated with the anti-GFP "miRNA", GFP fluorescence was not detected in the invading hyphae (**Figure 8,** middle panel). Most importantly, treatment of sheath leaves with anti-PMK1 simultaneously inoculated with fungal spores prevented hyphal growth in host cells. Instead, fluorescent spots scattered in the cytoplasm of the host cells were observed (**Figure 8,** lower panel). At the penetration site, a cell death reaction occurred (**Figure 8**, lower panel). A similar response was also observed in cells of excised rice sheaths upon infection with *M. oryzae* mutants defective for DES1 (*Defense Suppressor 1*) (Chi *et al.* 2009). DES1 is a pathogenicity gene that counteracts host defenses by compromising ROS-mediated plant defense responses. Susceptible rice plants infected by *Δdes1* mutant present accentuated hypersensitive defense responses seen as dark-brown precipitates at infected cells similar as we have reported (**Figure 8,** lower left panel).

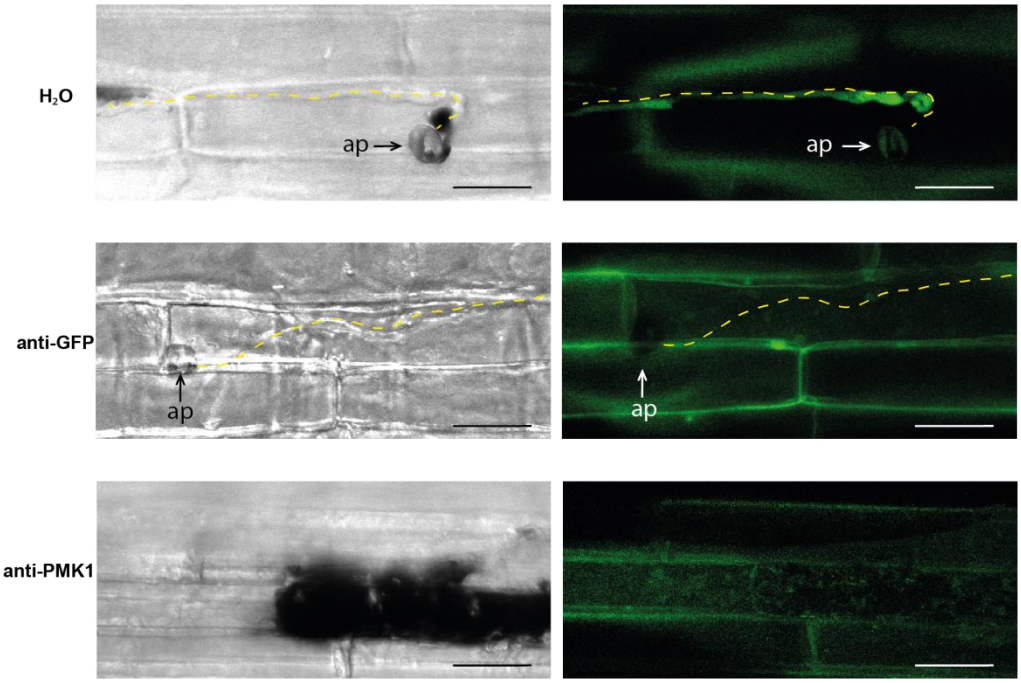


Figure 8. *M. oryzae* infection of leaf sheaths that have been treated with a miRNA precursor producing anti-PMK1 or anti-GFP. Leaf sheaths of 3 week-old rice plants were inoculated with the precursor miRNA (50 µl; 20 ng/µl) and *M. oryzae* spores (50 µl, 10^5 spores/ml). Plants were kept in a humid chamber at 28 ºC under dark conditions during 24 hours. Appressorium formation and penetration into the rice cells was evaluated by confocal microscopy at 24 hpi. Left panel, bright field. Right panel, GFP emission. Discontinuous yellow lines indicate *M. oryzae* hyphae. Specific *M. oryzae* GFP signal can be seen (upper panel) and non-specific rice autofluorescense (right panels). Scale bar: 20 µm. Ap, apressoria.

DISCUSSION

In this study, we provide evidence that exogenous application of miRNAs is effective for silencing genes in fungal pathogens, as an alternative to standard chemical fungicides used for the control of blast disease. In recent years, there has been an increasing number of publications indicating that cross-kingdom RNAi can be exploited in crop protection by generating transgenic plants expressing dsRNAs against selected gene targets in pathogens and pests (HIGS) (Zhao *et al.* 2021). Equally, the exogenous application of dsRNAs and/or siRNAs (SIGS) has proven to decrease disease caused by plant pathogenic fungi (i.e. *F. graminearum*, *B. cinerea*) (Koch *et al.* 2016; Wang *et al.* 2016b; Weiberg *et al.* 2013). Compared with HIGS, SIGS avoids host trangenesis. So far, no evidence has been provided on the use of miRNAs as potential antifungal agents through silencing of fungal genes. Our results demonstrated that exogenous application of miRNAs targeting *M. oryzae* genes essential for fungal development and/or pathogenesis can be used to protect rice plants from *M. oryzae* infection.

In this study, we investigated the capability of miRNAs to silence the expression of two *M. oryzae* genes, *MoPMK1* and *MoMAC1*. Three different methods for *in vitro* and *in vivo* preparation of miRNAs of interest were used for production of these miRNAs. The chemical synthesis allowed to synthetize pure small RNAs forming a miRNA duplex. Because the cost of chemical synthesis of RNAs is still a limiting factor for their application on a large scale, this approach was used as a "proof-of-concept" to assess the feasibility of using miRNAs for silencing target fungal genes. Transient expression in *N. benthamiana* leaves was used to produce miRNAs at a lower cost. To avoid gene silencing the *rdr6IR- N. benthamiana* line was used for the *in planta* production of miRNAs of interest. The production of precursor miRNAs was also approached by *in vitro* transcription.

We show that topical application of anti-PMK1 on rice leaves, either the chemically synthesized anti-PMK1, or the *in vivo* and *in vitro*

synthesized miRNA precursors, confers protection against *M. oryzae* infection in rice. Several lines of evidence support this conclusion. First, we show that topical application of anti-PMK1 significantly reduced blast lesions in rice leaves. Knowing that *MoPMK1* is fundamental for appressoria development, the observed reduction in blast symptoms in rice leaves could be attributed to interference on the signaling pathway for appressorium formation in anti-PMK1-treated rice leaves. Supporting this possibility, a dose-response relationship between the anti-PMK1 dose applied and the formation of appressoria was observed in this study. Additionally, *MoPMK1* is known to be implicated in cell-to-cell invasion by the rice blast fungus (Sakulkoo *et al.* 2018). In this study, infections experiments with a *gfp*-expressing *M. oryzae* isolated revealed that application of anti-PMK1 had a negative effect on growth of invasive hyphae. A pattern of fluorescent spots was observed suggesting degradation of the fungal cells in the invaded host cells. Also, a cell death reaction occurred at the host invaded rice cells. The antifungal effect of anti-PMK1 might well be the consequence of a combination of mechanism, interference with appressoria formation and induction of host defences at the infection sites. Further research is needed to assess whether anti-PMK1 application regulates the expression of defense-related responses in the rice plant.

On the other hand, we found that the application of *MoMAC1* did not result in a significant protection to *M. oryzae* infection. Clearly, the efficacy of strategies based on exogenous application of RNAs might be dependent not only on the target fungal gene, but also on the dose and stability of the RNA, time of application, and spatio-temporal expression pattern of the target gene. Whereas the effectiveness of anti-PMK1 in protecting rice from *M. oryzae* has been demonstrated, further studies are still required to ascertain whether anti-MAC1 is effective to prevent blast symptoms in other experimental conditions.

In recent years there has been increasing evidence on the important role of extracellular vesicles as vehicles for small RNA transport between plant and pathogens (Cai *et al.* 2019). During pathogen infection, pathogens can transport small RNAs into host cells to silence host defense

genes to suppress immunity, whereas hosts can deliver small RNAs into the pathogen to suppress infection. On the other hand, albeit uptake of external siRNA molecules by fungal cells has been observed, the mechanism by which RNA molecules enter into the fungal cells from the environment needs further investigation. This is the case of RNAs sprayed on plant surfaces which might be taken up directly by the fungal cells, or first taken up by the plant cells and then transferred into the fungal cells.

Another interesting finding of this study is that treatment with a miRNA was found to trigger systemic silencing of a fungal gene in rice leaves, indicating that miRNAs can be absorbed from the basis of an excised leaf to arrest pathogen infection in distal regions of the leaf. The rice leaves must have a mechanism to uptake and transport RNA molecules at distant sites. In other studies, rice plants were found to be able to absorb dsRNAs through the roots and trigger RNAi in stem-borer insect pests (Li *et al.* 2015). Furthermore, Koch and colleagues observed inhibition of *F. graminearum* growth in distal, non-sprayed barley leaf tissue (Koch *et al.* 2016). Although not proven, there is the possibility that RNAs might move systemically through vasculature. Delivery of miRNAs would be then particularly well-suited to protect crops grown under hydroponic conditions.

It is also true that, although the exogenous application of dsRNA and siRNA molecules has proven to be useful in reducing pathogen impacts on crop species, an important drawback of using long dsRNAs (and/or siRNAs derived from dsRNAs) is a possible lack of specificity of this strategy. As siRNAs derive from long, double-stranded RNAs that generate a diverse set of siRNAs, these siRNAs might lead to silencing undesirable genes. An advantage of using miRNAs for gene silencing is that artificial miRNAs can be tailored to be highly specific to direct silence the desired fungal gene. Also, miRNA-based fungicides could potentially be developed against specific pathogens and used in combination with other antifungal agents to increase the strength and/or duration of crop protection.

CONCLUSIONS

To conclude, results presented in this work demonstrated that exogenous application of miRNAs protects rice plants from the blast disease. However, there are still many aspects in the process of RNA silencing in plant-pathogen communication that remain largely unclear, including the mode by which small RNAs produced in one partner exploit the RNA silencing machinery in the partner organism. This aspect is particularly important when using miRNA precursors as antifungal agents, as miRNA precursors must be properly processed in fungal cells. In the case of *M. oryzae*, RNA silencing mechanisms were previously reported (Kadotani *et al.* 2003), but our knowledge on RNA silencing in phytopathogenic fungi is far less than that in plant species. This research might have important implications in understanding the biology of plant/pathogen interactions. From the practical point of view, several questions need to be addressed to eventually judge the potential of the application of miRNAs as antifungal agents, including the costs of production and their stability under field conditions. Before that, more research is required when the development of plant protection strategies based on miRNAs silencing of fungal genes is pursued.

MATERIALS AND METHODS

Chemical synthesis of small RNAs

Oligonucleotides were synthesized by successive additions of 2-cyanoethyl phosphoramidite derivatives on solid supports (1 micromol scale) using an automatic DNA/RNA synthesizer (Applied Biosystems 3400). To increase stability of small RNAs, the 5' end of each small RNA sequence was phosphorylated, while a 2'-O-methyl group was introduced at the 3' terminus using the chemical phosphorylation reagent (Horn and Urdea 1986) and the corresponding solid support functionalized with the appropriate 2'-O-methyl-RNA nucleoside (Sproat *et al.* 1989) both from Link Technologies (Scotland). The 2'-OH protecting group for the RNA monomers was the t-butyldimethylsilyl (TBDMS) group. Guanosine was protected with the dimethylaminomethylidene group, cytidine with the acetyl group and adenosine with the benzoyl group. The following ancillary solutions were used: 0.4M 1H-tetrazol in acetonitrile (ACN) (activation), 3% trichloroacetic acid in dichloromethane (DCM) (detritylation), acetic anhydride/pyridine/tetrahydrofurane (1:1:8) (capping A), 10% N-methylimidazole in tetrahydrofurane (capping B), 0.01M iodine in tetrahydrofurane/pyridine/ water (7:2:1) (oxidation). The average coupling yield was around 97–98% per step. The resulting supports were then treated with concentrated aqueous ammonia-ethanol (3:1) for 1 h at 55 ºC. The solid supports were filtered, washed with ethanol, and the combined filtrates were evaporated to dryness. Sequences were treated with 0.15 mL of triethylamine.tris(hydrofluoride)/ triethylamine/ N-methylpyrrolidone (4:3:6) for 2.5 h at 65 ºC to remove the TBDMS groups (Wincott *et al.* 1995). The reactions were stopped by adding 0.3 mL of isopropoxytrimethylsilane and 0.75 mL of ether. The resulting mixtures were stirred and cooled at 4 ºC. A precipitate was formed and then centrifuged at 7,000 rpm for 5 min at 4 ºC. The precipitates were washed with ether and centrifuged again. The residues were dissolved in water and the oligonucleotides were purified by HPLC (Aviñó *et al.* 2009). Column: Nucleosil 120-10 C18 (250 × 4 mm); 20

min linear gradient from 0% to 50% B (DMT-off conditions); flow rate 3mL/min; solution A was 5% ACN in 0.1M aqueous TEAA and B 70% ACN in 0.1M aqueous TEAA. The purified products were analyzed by MALDI-TOF mass spectrometry (Pourshahian 2021). MALDI-TOF spectra were obtained using a Perseptive Voyager DETMRP mass spectrometer, equipped with nitrogen laser at 337nm using a 3 ns pulse. The matrix used contained 2,4,6-trihydroxyacetophenone (THAP, 10 mg/mL in ACN/water 1:1) and ammonium citrate (50 mg/mL in water). Equimolar amounts of the two strands of the miRNA duplex were annealed using standard protocols.

Transient expression of miRNA precursors in *N. benthamiana* leaves

Template plasmid pNW55 (Warthmann *et al*. 2013), based on rice MIR528 precursor, was used for construction of the different miRNA precursors used in this work, (anti-PMK1, anti-MAC1 and anti-GFP miRNAs precursors, **Figure 2**). Oligonucleotides used for MIR528 substitution by specific anti-miRNA are listed in **Supplemental Table 2**. The PCR product was cloned into a pCR-Blunt vector (pCR-Blunt::*modifiedMIR528precursor*), and then was digested with BamHI/EcoRV and the precursor fragment was cloned into a BamHI/SmaI-digested binary expression vector under the control of the maize *ubiquitin1* promoter (*ZmUbi*) and the *nopaline synthase* (*nos*) terminator (*pC1300::ubi1::modifiedMIR528precursor::nos*). As a negative control, the empty vector was used (*pC1300*). The expression vectors were transferred to the *Agrobacterium tumefaciens* (GV3101 strain). The *N. benthamiana RDR6-IR* line, deficient in RNA-dependent RNA polymerase 6 was used in this study (Schwach *et al.* 2005). Leaves from 15-day-old *N. benthamiana RDR6-IR* plants were agroinfiltrated, and samples were harvested at 24 hpi and 96 hpi. The leaf tissues were stored at -80ºC until use.

In vitro synthesis of miRNA precursors

The pCR-Blunt*::modifiedMIR528precursor* vectors (anti-PMK1, anti-MAC1 or anti-GFP miRNAs precursors) obtained as described before, were used for *in vitro* transcription of miRNA precursors, as the pCR-Blunt vector contains the sp6/T7 promoter. Plasmids containing the miRNA precursor under study were linearized with the restriction enzyme *Spe*I. The *in vitro* transcription was carried out with T7 RNA polymerase following the manufacture instructions. Briefly, the reaction mixture containing 1 µg of linearized DNA template was incubated at 37ºC for 16 hours followed by treatment with TURBO DNase, Phenol:chloroform extraction and isopropanol precipitation. RNA concentration and purity was confirmed by spectrophotometry (NanoDrop, ND-1000) and RNA size was confirmed by Bioanalyzer gel (Agilent 2100 Bioanalyzer System).

Expression analyses

Total RNA was extracted from plant tissues by using TRizol reagent (Invitrogen). The small RNA fraction was purified by PEG8000/NaCl precipitation. Briefly, 50 µg of RNA were mixed (1:1) with a solution containing PEG8000 (20%) and NaCl (1M). Samples were left 4 hours in ice and centrifuged during 15 minutes at 13.000 rpm at 4ºC. The supernatant containing the low molecular weight RNAs was recovered. The RNA was precipitated by adding 2.5 volumes of absolute ethanol and 0.1 volumes Sodium Acetate 3M. Samples were left at -20ºC overnight. Then, the RNA was centrifuged for 15 minutes at 13.000 rpm at 4ºC. Supernatant was discarded and the pellet was cleaned with ethanol 75%. After 5 minutes centrifugation at 13.000 rpm at 4ºC the supernatant was removed and RNA was air dried for no more than 5 minutes. Samples were resuspended in 20 µl of autoclaved distilled water. For northern blot analysis, small miRNAs were fractionated on 17.5% denaturing polyacrylamide gel containing 8M urea, transferred to nylon membranes,

and probed with a γ ^{32}P-ATP- labeled oligonucleotide probes complementary to the "miRNA" of interest (**Supplemental Table S2**). Blots were pre-hybridized and hybridized in Perfect-Hyb Plus buffer (Sigma) at 42 °C. Hybridization signals were detected by using STORM Phosphorimager (GE Healthcare).

For quantitative RT-PCR (RT-qPCR), the first complementary DNA was synthesized from DNase-treated total RNA (1 µg) with High Capacity cDNA Reverse Transcription (Life technology, Applied Biosystems). Primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) at miRNA flanking sites. RT-qPCR was carried out in optical 384-well plates (Roche Light Cycler 480; Roche Diagnostics, Mannheim, Germany) using SYBR Green I dye and gene-specific primers (**Supplemental Table S2**). The *M. oryzae Tubuline* gene (Skamnioti and Gurr 2007) was used to normalize transcript levels.

The accumulation of anti-PMK1, anti-MAC1 and anti-GFP small RNA sequences was determined by stem-loop RT-qPCR (Varkonyi-Gasic *et al.* 2007). Primers are listed in **Supplemental Table S2**.

Infection assays

Rice plants *O. sativa* var. Nipponbare were grown at 28 °C with a 14 h/10 h light/ dark cycle. *M. oryzae* was grown in Complete Media Agar (CMA, 9 cm plates, containing 30 mg/L chloramphenicol) for 15 days at 28ºC under a 16 h/8 h light/dark photoperiod condition. *M. oryzae* spores were prepared as previously described (Campo *et al.* 2013).

Infection assays with the rice blast fungus *M. oryzae* (PR9 strain) were carried out using the detached leaf assay as previously described (Coca *et al.* 2004). For this, the youngest fully developed leaf of soil-grown rice plants at the 3-4 leaf stage was placed on 1% agar plates (10 leaves/ plate, square plants) containing kinetin (2 mg/l). The miRNA under study was

applied on the adaxial leaf surface at the specified concentration. After 15 minutes of miRNA treatment, a whatman filter paper disc saturated with a *M. oryzae* spore suspension (5 x 10^5 spores/ml) was placed onto the RNA-treated area, and maintained for 60 h. The percentage of leaf area affected by blast lesions was determined at 6 days post-inoculation with *M. oryzae* spores by using the APS Assess 2.0 program (Lamari 2008).

Local infections were performed as described (Coca *et al.* 2004). For apressoria counting, the rice leaves were treated with the appropriate miRNA, or water-treated and kept under high humidity conditions. Leaves were then locally inoculated with *M. oryzae* spores and visualized under bright field conditions 16 hours post inoculation. A total of 40 inoculated regions from four independent leaves were examined.

Infection experiments on leaf sheaths were carried out as described by Jones and Khang (2018) using a *gfp*-expressing *M. oryzae* strain previously characterized (gfp-*M. oryzae*, PR9 strain) (Campos-Soriano *et al.* 2013; Campos-Soriano and San Segundo 2009).

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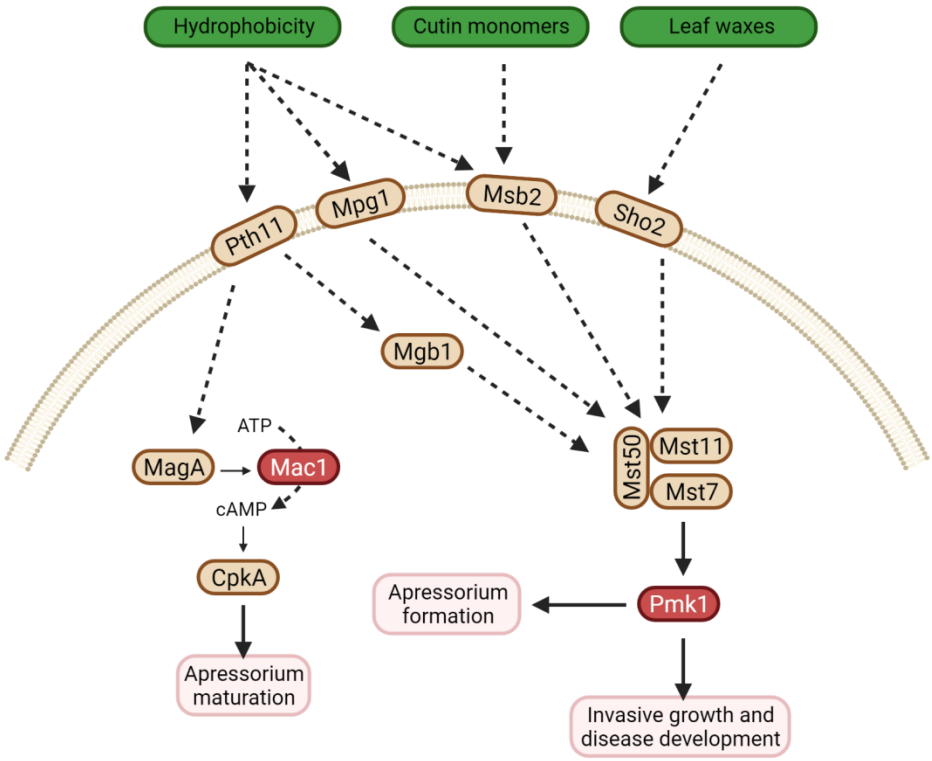
SUPPLEMENTAL FIGURES



Supplemental Figure S1. Schematic *MoPMK1* and *MoMAC1* signaling pathway. **CpkA**, cAMP-dependent protein kinase catalytic subunit; **Mac1**, adenylate cyclase; **MagA**, Gα subunit; **Mgb1**, Gβ subunit; **Mpg1**, hydrophobin-like protein; **Msb2**, signaling mucin; **Mst7, Mst11, Mst50**, Mitogen-activated protein kinase adapter proteins, 7, 11 and 50; **Pmk1**, mitogen-activated protein kinase; **Pth11**, G-protein-coupled membrane receptor; **Sho2**, high osmolarity signaling protein 2.

>MGG_09898 Magnaporthe grisea adenylate cyclase (MAC1) gene, complete cds

ATGACCAGGAACGACGCCCTGTCCCGTTTCAGCTCCATTACGAGCTCCTCCACAGACAGTGCACACTCAACGGCTACCGTTAAGCCGTTGCCATCAACGCCTATCGCCAGCGATACACGAAGACATTCA
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>MGG_09565 Magnaporthe grisea pathogenicity MAP kinase 1 (PMK1) gene, complete cds.

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>GFP cDNA (from M. oryzae-GFP; pCAMgfp plasmid

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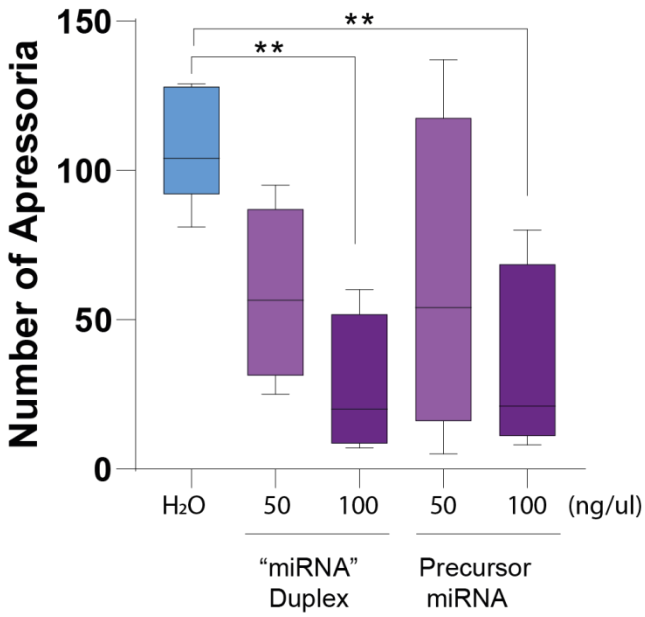
Supplemental Figure S2. Complete CDS of *M. oryzae MoMAC1*, *MoPMK1* and *GFP*. MiRNA recognition sites are shaded in yellow.



Supplemental Figure S3. Dose response in anti-PMK1 treated plants. Rice leaves were treated as in Figure 7A, but using a solution of RNA at 100 ng/µl. Data are mean ± SEM (4 biological replicates). Asterisks indicate statistically significant differences calculated by one way ANOVA **, $p \leq 0.01$).

Target Score	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	
M. oryzae	1				1		4	12	1			anti-PMK1
O. sativa (RAPDB)						3	6	10				
O. sativa (MSU)						7	8	3				
M. oryzae	1						3	2	7	7		anti-MAC1
O. sativa (RAPDB)					1		6	9				
O. sativa (TIGR)					1		6	12				
M. oryzae							1	1	9	9		anti-GFP
O. sativa (RAPDB)					1	1	13	3				
O. sativa (MSU)					1	1	15	3				

Supplemental Table S1. miRNA target score analysis. The target score was calculated using the pSRNAtarget tool (https://plantgrn.noble.org/psRNATarget/) against *M. oryzae* genome or *O. sativa* genome (MSU or RAPDB annotations), and represented from 0 to 5 (green, good targets; yellow, acceptable targets; red, bad targets). Numbers in each box correspond to the number of targets found for each target score in each one of the represented species. Shaded green boxes refer to *MoPMK1* (mitogen-activated protein kinase1) and *MoMAC1* (adenylate cyclase1) genes, or the GFP (green fluorescent protein) gene. Genes with low scores are recommended. Possible off-targets (light shaded boxes) need to be in the yellow or red zone (red better).

Oligo Name	Sequence	Gene name	Figure
anti-PMK1_Fw_RNA	UCAAUAGCCUUGGUGUGCUCC	anti-PMK1 "miRNA"	Fig. 1
anti-PMK1_Rv_RNA	AGCUCACGAAGGCUAUUGAU		
anti-MAC1_Fw_RNA	UUGUUUGCCCACCAAAUCUCC	anti-MAC1 "miRNA"	
anti-MAC1_Rv_RNA	AGAAUUGCUGGGCAAACAAUU		
anti-GFP_Fw_RNA	UCGCCGGACACGCUGAACUUG	anti-GFP "miRNA"	
anti-GFP_Rv_RNA	AGUACAGGGUGUCCGGCGAUU		
SC329-PMK1	AATCAATAGCCTTCGTGAGCTCC	anti-PMK1 northern probe	Fig. 3B
SC331-MAC1	AATTGTTTGCCCAGCAATTCTCC	anti-MAC1 northern probe	
SC333-GFP	AATCGCCGGACACCCTGTACTTG	anti-GFP northern probe	
FS_PMK1_RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAGCA	anti-PMK1 stem-loop	Fig. 3C
FS_PMK1_qPCR_Fw	GGCGGTCAATAGCCTTGGTG		
FS_MAC1_RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAGAT	anti-MAC1 stem-loop	
FS_MAC1_qPCR_Fw	GGCGGTTGTTTGCCCACCAA		
FS_GFP_RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGTT	anti-MAC1 stem-loop	
FS_GFP_qPCR_Fw	GGCGGTCGCCGGACACGCTG		
FS_Mo_MAC1_Fw	GCTCAAATTCCTCGATGTGC	*MoMAC1*	Fig. 4A, D; Fig. 5B
FS_Mo_MAC1_Rv	ATAGAACCGGGACTCGACTC		
FS_MoPMK1_Fw	CATGACAGAATACGTCGCTAC	*MoPMK1*	
FS_MoPMK1_Rv	TTTCAGCCAGAATGCAGCC		
FS_Mo_bTUB_Fw	CTCTGCCATCTTCCGTGGA	*MobTUB*	
FS_Mo_bTUB_Rv	ACGAAGTACGACGAGTTCTTGTTCT		
SC308-G-11491-mod	TCGGATCCCAGCAGCAGCCACAGCAAA	*cloning pC1300::ubi1::modifiedMIR528::nos*	
SC309-G-11494-mod	ggGATATCGGTACCGCTGCTGATGCTGATGCCAT		
SC310-I_antiPMK1-s	agTCAATAGCCTTGGTGTGCTCCcaggagattcagtttga		
SC311-II_antiPMK1-a	tgGGAGCACACCAAGGCTATTGActgctgctgctacagcc		
SC312-III_antiPMK1*s	ctGGAGCTCACGAAGGCTATTGAttcctgctgctaggctg		
SC313-IV_antiPMK1*a	aaTCAATAGCCTTCGTGAGCTCCagagaggcaaaagtgaa		
SC314-I_antiMAC1-s	agTTGTTTGCCCACCAAATCTCCcaggagattcagtttga		
SC315-II_antiMAC1-a	tgGGAGATTTGGTGGGCAAACAActgctgctgctacagcc		
SC316-III_antiMAC1*s	ctGGAGAATTGCTGGGCAAACAAttcctgctgctaggctg		
SC317-IV_antiMAC1*a	aaTTGTTTGCCCAGCAATTCTCCagagaggcaaaagtgaa		
SC318-I_antiGFP-s	agTCGCCGGACACGCTGAACTTGcaggagattcagtttga		
SC319-II_antiGFP-a	tgCAAGTTCAGCGTGTCCGGCGActgctgctgctacagcc		
SC320-III_antiGFP*s	ctCAAGTACAGGGTGTCCGGCGAttcctgctgctaggctg		
SC321-IV_antiGFP*a	aaTCGCCGGACACCCTGTACTTGagagaggcaaaagtgaa		

Supplemental Table S2. Oligonucleotides used in this study.

General discussion

During the last years, significant advances have been made in our knowledge on components and regulatory mechanisms involved in plant immunity. It is also true that most of these studies focused on transcriptional reprogramming of gene expression during pathogen infection. There is also evidence that miRNAs play an important role in controlling plant immunity in different plant species. These riboregulators can play positive or negative roles in regulating plant immune responses. Current research in our group is devoted to the elucidation of miRNA-mediated regulatory processes underlying disease resistance in rice plants, and cross-talk between plant immunity and nutrient stress.

In this study, I sought to better understand mechanisms involved in the response of rice plants to pathogen infection in which miR7695 participates, as well as cross-talk between iron signaling pathways and immune signaling pathways. These studies have been carried out in the interaction of rice (*O. sativa* spp *japonica*) with the fungus *M. oryzae*, one of the most important pathogens affecting rice production worldwide. In addition to its agricultural relevance, rice has been adopted as the model plant for functional genomics research in monocotyledoneous plant species.

A major outcome of this research project relates to the characterization of the molecular and cellular mechanisms by which miR7695 and its target gene *OsNramp6* integrates iron signaling and pathogen-induced signaling pathways. Results obtained in this research are included in **Chapter 1** and **Chapter 2** of this thesis. From a practical perspective, I also investigated the feasibility of using miRNAs antifungal agents for protection of rice plants against the blast fungus. Results obtained in this study are presented in **Chapter 3**.

In previous studies, our group identified miR7695 as a young, recently evolved miRNA from rice that experienced natural and domestication selection during evolution (Campo *et al.* 2013). miR7695 negatively regulates the expression of the shortest transcript variant of *OsNramp6* encoding a plasma membrane iron transporter (Campo *et al.* 2013; Peris-peris *et al.* 2017). Changes in the accumulation of miR7695

and *OsNramp6* transcripts (full-length and short transcripts) occur during *M. oryzae* infection and treatment with *M. oryzae* elicitors (Campo *et al.* 2013; Peris-peris *et al.* 2017). Both the full-length and the shortest NRAMP6 proteins (the later one being the miR7695 target), exhibit iron transport activity and plasma membrane localization (Peris-peris *et al.* 2017). Most probably, miR7695 plays a role in controlling the relative abundance of full-length and short *Nramp6* transcript variants. This regulatory network would integrate miRNA function and mRNA processing for the control of *OsNramp6* expression. Importantly, *MIR7695* overexpression and loss-of-function of *Nramp6* were found to enhance blast resistance in rice (Campo *et al.* 2013; Peris-peris *et al.* 2017). This piece of evidence already indicated that miR7695 plays a role in resistance to infection by the rice blast fungus. On this basis, in this Ph. D., I investigated the mechanisms through which miR7695, and its target gene *Nramp6*, regulate immune responses, and how iron can impact these responses.

Studies on activation-tagged *MIR7695* rice plants (*MIR7695*-Ac) demonstrated that *MIR7695-Ac* plants had a super-induction of defense-related genes compared with wild-type plants during infection, including pathogenesis-related and diterpenoid biosynthetic genes. Phytoalexins accumulated at a high level during infection of *MIR7695-Ac*. Along with this, *MIR7695*-Ac plants exhibited resistance to *M. oryzae* infection (Sánchez-Sanuy *et al.* 2019) (**Chapter I**). As it was observed in *MIR7695-Ac* plants, *Osnramp6* mutant plants also showed blast resistance (Peris-peris *et al.* 2017). These results indicated that miR7695 is a positive regulator of defense responses in rice.

Different studies were performed to ascertain the biological function of miR7695 and *Nramp6* in rice immunity. They were carried out on *japonica* rice cultivars (*O. sativa* Nipponbare, Tainung 67, Hwayoung). According to our results, accumulation of miR7695 species and/or reduction of *OsNramp6* transcripts is accompanied by blast resistance. In this respect, a comparative transcriptome profiling of *japonica* cultivars with contrasting response to blast infection (Gigante

Vercelli, highly resistant; Vialone nano, highly susceptible; both varieties being cultivated in Italy) revealed a weaker induction of *OsNramp6* expression in the resistant genotype (Gigante Vercelli) compared with the susceptible genotype (Vialone nano) (Bagnaresi *et al.* 2012). More recently, a stronger induction of *MIR7695* expression during *M. oryzae* infection was associated to blast resistance in *indica* varieties compared with that in blast-susceptible varieties (varieties cultivated in Vietnam) (Quoc *et al.* 2019). This fact correlates well with the observed reduction of *Nramp6* expression in the blast resistant Vietnamese rice cultivars compared with the susceptible ones during *M. oryzae* infection (Phi et al 2021). Results obtained in this Ph. D. Thesis (Peris-peris *et al.* 2017; Sánchez-Sanuy *et al.* 2019) are in accordance with those found in the literature on Italian and Vietnamese rice cultivars and highlight the potential of *Osa-MIR7695* and/or *OsNramp6* as biomarkers for blast resistance in rice breeding programs.

Treatment of wild-type plants with high Fe was found to confer resistance to *M. oryzae* further illustrating a close link between Fe availability and rice immunity. The protective effect of iron treatment was observed in both hydroponically-grown plants and soil-grown plants (Peris-peris *et al.* 2017; **Chapter I** and **Chapter II**). Histochemical detection of Fe in *M. oryzae*-infected rice leaves revealed Fe accumulation at the sites of attempted penetration by the fungus (appressoria) and cells in the vicinity of the infection sites (Sánchez-Sanuy *et al.* 2019; **Chapter II**). This localized accumulation of iron was observed in *MIR7695-Ac* plants as well as in wild-type plants that have been treated with high Fe (**Chapter I** and **Chapter II**). Additionally, double staining of *M. oryzae*-infected leaves revealed accumulation of both Fe and ROS at the sites of pathogen penetration in wild-type plants that have been treated with high iron (**Chapter II**). As ROS production is a ubiquitous response to pathogen infection in plants, and iron is a mediator of ROS production (Fenton reaction), it can be concluded that Fe accumulation provokes ROS accumulation at the infection sites. In line with this, Dangol and collaborators (2019) demonstrated that infection of

rice plants with an avirulent *M. oryzae* strain provokes an iron-dependent cell death process, or ferroptosis, resulting in blast resistance. However, infection of rice plants with a virulent *M. oryzae* strain did not induce ferroptosis, resulting in blast susceptibility. In this way, treatment of wild-type rice plants with high Fe might facilitate ferroptotic cell death to limit pathogen invasion, as it was previously observed during an incompatible rice/*M. oryzae* interaction (Dangol *et al.* 2019).

Treatment with high Fe in wild-type plants as well as *MIR7695* activation *(MIR7695-Ac* plants*)* is accompanied by stronger induction of defense gene expression during pathogen infection (e.g. *PR* genes and phytoalexin biosynthesis genes). The observed superinduction of phytoalexin biosynthesis genes in Fe-treated wild-type plants and *MIR7695-Ac* plants is consistent with a higher production of phytoalexins in these plants during *M. oryzae* infection, diterpene phytoalexins and sakuranetin in the case of Fe-treated wild-type plants, and diterpene phytoalexins in the case of *MIR7695-Ac* (here the accumulation of sakuretine was not examined). We also show that, under the experimental conditions assayed, Fe treatment had a low impact on the global transcriptome of rice leaves and that, only upon pathogen infection, the plant responds with a more robust defense reaction. These observations rise a number of interesting questions on how Fe-treated rice plants responds to infection with a more robust defense reaction (e.g. superinduction of *PR* and phytoalexin biosynthesis genes). It is tempting to hypothesize that high Fe is perceived by the plant as a stressful situation which triggers stronger induction of defense related genes during pathogen infection. The observed stronger induction of defense-related genes in *MIR7695-Ac* and Fe-treated is reminiscent of defense priming, a phenomenon associated with different forms of induced resistance to pathogen infection based on faster and/or stronger activation of defense responses. While a stronger activation of defense responses by Fe treatment has been demonstrated in this work, further studies are required to investigate whether Fe-treated rice plants also activated defense faster

than non-treated rice plants. An interplay between Fe- and pathogen-induced signal transduction pathways must occur in rice.

Results presented in **Chapter II** revealed that under normal growth conditions, Fe treatment was accompanied by down-regulation of genes that are typically induced by Fe deficiency in rice leaves, which is in agreement with the observed increase in Fe content (total Fe and apoplastic Fe) in leaves of plants that have been treated with high Fe. It is noteworthy that *M. oryzae* infection was also found to provoke alterations in leaf Fe content. Upon pathogen infection, there is a decrease in total Fe and apoplastic Fe content. The observed decrease in total Fe content due to *M. oryzae* infection can be attributed to a reduction in ferric ions (Fe^{3+}). Although a reduction of Fe occurs in *M.oryzae*-infected leaves, it is unlikely that this reduction might have a drastic effect on *M. oryzae* growth. The rice plant appears not to use an iron-withholding strategy to arrest *M. oryzae* infection. Further investigation is however needed to clarify this aspect.

To provide further insights in the relationship between iron accumulation in blast resistance, I planned to perform a stage in the laboratory of Dr. Jian Feng Ma (Okayama University, Japan). In 2020, I was granted with a Short-term EMBO fellowship for a visit to Dr. Ma Plant Stress Physiology Laboratory early in 2021. For this stage, experiments were proposed to monitor iron in different tissues and cellular sub-compartments by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) in *M. oryzae* infected rice plants (iron-treated wild-type plants, *MIR7695*-Ac plants and *nramp6* mutant plants). Due to the coronavirus pandemic my visit to the laboratory in Japan had to be cancelled.

Furthermore, during the course of studies presented in Chapter I and Chapter II, I generated CRISPR-Cas9-edited *Nramp6* mutant lines using a double guide RNA system (results not included in this Thesis). Silencing of *OsNramp6* has been confirmed in these lines. Preliminary experiments carried out in CRISPR/Cas9-edited *nramp6* mutants at the T2 generation

(lines harbouring homozygous mutations) revealed resistance to *M. oryzae* infection in these lines.

Collectively, the information gained in Chapter I and Chapter II in this Ph. D. Thesis provided new insights into the molecular mechanisms that operate in rice immunity, while demonstrating the important role of miR7695 and is target gene *Nramp6* in regulating iron homeostasis and immune signaling. These studies also demonstrated that the iron nutritional status of rice plants strongly affects blast resistance. In other studies, it has long been recognized that excess of nitrogen increases susceptibility to infection by the rice blast fungus, the so called Nitrogen-induced susceptibility (NIS) to blast (Ballini *et al.* 2013). More recently, our group reported that high phosphate supply enhances susceptibility to *M. oryzae* in rice plants (Campos-Soriano *et al.* 2020). In contrast, results here presented indicated that high Fe supply increases blast resistance. Then, signaling pathways controlling the plant response to pathogen infection under nutrient stress may interact in a positive or negative manner with immune signaling depending on the type of nutrient. The impact of nutrient stress on disease resistance is difficult to predict as different results are found in the literature depending on the host plant and the type of pathogen. Because *M. oryzae* infection causes major problems in rice cultivation, is of paramount importance to investigate the impact of the nutrient status of the rice plant on blast resistance. The nature of such interactions deserves further investigation.

Based on the expertise and knowledge gained from studies on miRNAs, during this Ph. D. Thesis I participated in a more applied research project aiming to evaluate the utility of miRNAs as antifungal agents for protection of rice plants against the blast fungus (**Chapter III**). These studies demonstrated that topical application of miRNAs targeting key virulence fungal genes protects the rice plant from *M. oryzae* infection. Albeit the external application of siRNA molecules has proven to be effective to provide resistance to pathogen infection in different plant/fungal pathogen interactions, at present, no information is available

in the literature on silencing of fungal genes by the topical application of a miRNA onto a plant tissue.

Based on results obtained in this study, either miRNAs (e.g. synthetic RNAs forming a miRNA duplex) or precursor miRNAs, when applied on rice leaves, direct silencing of a fungal gene (as demonstrated by silencing of *MoPMK1* expression). However, the application of miRNA-based strategies requires a rational design of miRNA molecules. The success of a miRNA-based strategy aiming to silence fungal genes relies on selection of appropriate target genes in the fungal pathogen. Thus, whereas the application of anti-PMK1 inhibited pathogen growth, the application of anti-MAC1 was not effective to protect the rice plant from *M. oryzae* infection. There are still many aspects that need to be addressed to understand the mechanisms underlying silencing of fungal genes through external application of small RNAs (siRNAs, miRNAs). For instance, the RNAs sprayed on plant surfaces might be taken up directly by the fungal cells, or they can be first taken up by the plant cells and then transferred into the fungal cells. The effectiveness and long-term durability of miRNA-based strategies can be improved by developing formulations (i.e. modified miRNA molecules, nanoparticle-encapsulated miRNAs, lipid-vesicles, etc) that might increase stability or delivery of RNA molecules.

Using miRNAs for protection against fungal disease might represent an alternative to strategies based on the use of siRNAs for crop protection, namely HIGS and SIGS. The HIGS involves transgenic expression of dsRNAs targeting genes in the interacting pathogen. The use of GM plants is, however, banned in many countries. The SIGS involves inhibition of pathogen growth through direct spray of dsRNAs or siRNAs targeting pathogens on plant tissues. A possible risk of strategies based on spraying dsRNAs is the lack of specificity (compared to miRNAs).

An important challenge when using RNAs for external application in crop protection is the cost of this approach: chemical synthesis of RNAs is expensive nowadays. This situation is, however, changing rapidly (few years ago it was much more costly to produce synthetic RNAs). Indeed, the success observed on the application of siRNAs in crop protection, moved companies in the agribusiness sector to exploit existing and emerging technologies for mass production of dsRNAs at low cost (Taning *et al.* 2020).

For future applications of miRNAs in crop protection, there are still many issues that need to be investigated in the following directions: i) miRNA stability and long term effect application; ii) possible off-target effects of miRNA application in the pathogen or in the host plants. Application of miRNAs might also have effects in other organisms, hence, miRNA sequences must be carefully designed to avoid undesired effects on ecological communities. These studies are clearly required for the development of an innovative and safe technology based on miRNAs as antifungal agents.

Finally, in the course of this Thesis, I contributed to the functional characterization of a new member of the miR812 family of rice miRNAs in the context of resistance to infection by the rice blast fungus *M. oryzae*. The results derived from these studies are included in a recent publication (Campo *et al.* 2021. Plant Biotechnology J. 2021; 19:1798-1811; available online). In this work, we provided evidence that miR812w, a new member of the rice miR812 family, plays a regulatory role in blast resistance. Whereas *MIR812w* overexpression increases blast resistance, CRISPR/Cas9-mediated *MIR812w* editing enhances disease susceptibility. We described that miR812w derives from the *Stowaway* type of rice MITEs (Miniature Inverted-Repeat Transposable Elements). MiR812w directs DNA methylation in *trans* at target genes that have integrated a Stowaway MITE copy (i.e. *ACO3, CIPK10,* and *LRR* genes), as well as in *cis* at the *MIR812w* locus. These findings support that, in addition to post-transcriptional regulation of gene expression, miRNAs can exert their regulatory function at the transcriptional level.

A better understanding of gene regulatory networks in which miRNAs participate will provide useful tools for developing novel strategies for crop protection. Results obtained in this Ph. D. Thesis also highlight environmental modulation of plant immunity (e.g. nutrient supply), illustrating nodes between biotic and abiotic stress signaling pathways. Clearly, plants must coordinate conflicting responses when exposed to combined stresses, an aspect that deserves further investigation.

Conclusions

1. The iron nutritional status of rice plants has an effect on resistance to infection by the blast fungus *M. oryzae*. Treatment of wild-type plants with high Fe triggers superactivation of defense related gene expression during pathogen infection, which is consistent with a defense priming effect. This might well explain the observed phenotype of blast resistance in iron-treated rice plants.

2. *MIR7695* activation is also accompanied by transcriptional priming in defense responses against the rice blast fungus *M. oryzae* and blast resistance, indicating that miR7695 is a positive regulator of rice immunity. Similarly, silencing of the miR7696-targeted gene *OsNramp6*, which encodes an iron transporter, confers resistance to infection by *M. oryzae*. Together, these observations support the existence of molecular interactions between iron signaling and immune signaling, a process in which miR7695 and *OsNramp6* participate.

3. *MIR7695* activation as well as treatment of wild-type plants with high iron is accompanied by a higher production of phytoalexins during *M. oryzae* infection, both diterpene phytoalexins and the flavonoid phytoalexin sakuranetin. Interconnected regulations between iron homeostasis and phytoalexin biosynthesis must exist but the exact mechanisms of this regulation are as yet unknown.

4. During *M. oryzae* infection, iron and H_2O_2 accumulate in cells in the vicinity of the invading pathogen in wild-type plants grown with high Fe supply. A process of ferroptosis might then limit pathogen

invasion in iron-treated rice plants, a process that is known to occur in rice plants infected with an avirulent *M. oryzae* strain.

5. The total iron content increases in leaves of *M. oryzae*-infected rice plants grown under normal Fe supply, while its level slightly decreases in *M. oryzae*-infected plants grown in high Fe. A reduction in the content of ferric ions is also observed in the *M. oryzae*-infected leaves of high-Fe rice plants. Furthermore, *M. oryzae* infection causes a decrease in apoplastic iron content in high-Fe rice plants in which levels are similar to those observed in non-treated plants. It is unlikely that an iron-withholding strategy operates in high-Fe plants rice plant aiming to deprive the pathogen of this nutrient.

6. Tailor-made miRNAs designed to silence fungal genes with a relevant function in pathogenesis represent a new tool for crop protection, in particular for protection of rice plants against the rice blast fungus.

7. The application of RNAs, either synthetic RNAs forming a miRNA duplex, or precursor miRNAs, designed to silence *MoPMK1* expression might be especially useful for the control of the blast disease. The protective effect of anti-PMK1 against *M. oryzae* infection occurs both locally and systemically in rice leaves. Results obtained in these studies open new avenues for agronomical applications of tailor-made miRNAs to improve disease resistance in crops.

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