Universitat Autònoma de Barcelona

Crosstalk between phosphate signaling pathways and immune responses in plants

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Memoria presentada por Beatriz Val Torregrosa para optar al título de Doctor por la Universidad Autónoma de Barcelona.

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A mi padre…

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Abbreviations

ABA	Abscisic acid
ACO	1-aminocyclopropane-1-carboxylase oxidase
AFB	Auxin Signaling F-Box
AGO	ARGONAUTE
AP2	APETALA2
ARF	Auxin Response Factor
CDPK	Calcium-dependent protein kinase
CDS	Cythidinaphpsphate Diacylglycerol Synthase3
CIPK	Calcineurin B Like (CBL)-interacting serine– threonine protein kinase
CRISPR	Clustered regulatory interspaced short palindromic repeat
CSD	Copper superoxide dismutases
cv.	cultivar
DAMP	Damage associated molecular pattern
DCL	Dicer-like
dpi	Days post infection
EIHM	Extra invasive hyphal membrane
EIN2	Ethylene insensitive 2
ET	Ethylene
ERF	Ethylene response factor 1
ETI	Effector triggered immunity
Flg22	Flagellin 22
GRF	Growth-regulating factor
H_2O_2	Hydrogen peroxide
HEN1	HUA ENANCER 1
Hpi	Hours post infection
HR	Hypersensitive response
HST	HASTY
IH	Invasive hyphae
IPS1	Induced by phosphate starvation 1
JA	Jasmonic acid
MAPK	Mitogen-activated protein kinase
Mb	Mega base
MET2	Methyltransferase 2
miRNA	MicroRNA
mRNA	Messenger RNA
MYB	V-myb myeloblastosis viral oncogene homolog
NAC	No apical meristem

NF-YA	Nuclear transcription factor Y subunit alpha
NLA	Nitrogen limitation adaptation
NPR1	Non-expressor of pathogenesis-related 1
NRAMP6	Natural resistance-associated macrophage protein 6
O^{2-}	Superoxide ion
OH^{-}	Hydroxyl radical
PAM	Protospacer adjacent motif
PAMP	Pathogen associated molecular pattern
Pd	Plasmodesmata
PDF1.2	Plant defensing 1.2
phasiRNA	Phased RNA
PHF1	Phosphate transporter traffic facilitator
PHO2	Phosphate 2
PHT1	Phosphate transporter 1
PHR1/2	Phosphate regulator 1/2
PP	Penetration peg
PR protein	Pathogenesis related protein
Pre-miRNA	miRNA precursor
Pri-mRNA	Primary miRNA
PRR	Pattern recognition receptor
PTI	PAMP triggered immunity
pv.	Pathovar
RBOH	Respiratory burst oxidase homologue
R proteins	Resistance proteins
RISC	RNA induced silencing complex
ROS	Reactive oxygen specie
RPS5	Resistant to Pseudomonas syringe
RT-PCR	Reverse transcriptase-polymerase chain reaction
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
SA	Salicylic acid
sgRNA	Single-guide RNA
siRNA	Small-interfering RNA
SOD	Superoxide dismutase
SPL	SQUAMOSA promoter-binding protein-like
sRNA	Small RNA
TCP	Teosinte Branched1, Cycloidea, and Proliferating Cell Nuclear Antigen
TIR	Transport inhibitor response 1-like protein
VSP2	Vegetative storage protein 2
WT	Wild type

Summary

In nature, plants are constantly confronted to both abiotic and biotic stresses that seriously reduce their productivity. Research so far carried out to understand the plant response to biotic and abiotic stress has mainly concentrated on individual stresses. One possible outcome of exposure to a combination of stresses is that the plant defense response to a particular stress might have a positive or negative effect in the response to other type of stress due to crosstalk between their respective signaling pathways.

In the course of evolution, plants have evolved complex mechanisms allowing them to arrest pathogen infection. Currently, there is a large body of information and data in the literature on the transcriptional reprogramming of gene expression in plants during pathogen infection. Evidence also supports that plant immune responses can be posttranscriptionally regulated by the activity of microRNAs (miRNAs). Limited information is, however, available on miRNAs participating in the crosstalk between pathogen-induced signaling pathways and nutrient signaling in plants. On the other hand, nutrient supply (e.g. fertilization) has been shown to have an impact on resistance to pathogen infection in agricultural ecosystems.

Phosphorus is an essential nutrient for plant growth and development. Plants acquire this nutrient from the soil in the form of inorganic phosphate (Pi). The low bioavailability of Pi in agricultural soils represents a limiting factor for plant growth. As a consequence, Pi fertilizers are routinely used in modern agriculture to optimize crop yields, leading to a scenario of Pi excess in many agricultural ecosystems. At the same time, pesticides are commonly used for crop protection against pathogen infection. Excessive use of fertilizers and pesticides has a negative impact on human health and the environment. At the molecular level, a large effort has been made to understand how plants adapt to Pi limiting conditions through the so called Phosphate Starvation Response (PSR). Two miRNAs, miR399 and miR827, are important players in the regulation of Pi homeostasis and adaptation to Pi starvation conditions in plants.

Less information is available on adaptive mechanisms to Pi excess in plants. The main topic in this PhD Thesis is the investigation of interconnected regulations between Pi homeostasis and immune responses in Arabidopsis (Chapter I and Chapter II) and rice (Chapter III). Specifically, miR399- and miR827-mediated regulation of phosphate homeostasis and disease resistance has been investigated in this PhD Thesis.

Chapter I describes the function of miR399 and its target gene *PHO2* (PHOSPHATE2) in the regulation of phosphate homeostasis and disease resistance in Arabidopsis. Fungal pathogens used were: *Plectosphaerella cucumerina* (necrotroph) and *Colletotrichum* (hemibiotroph). Results obtained in this study indicated that growing plants grown under high Pi supply, as well as miR399 overexpression and loss-of-function of *PHO2* plants, leads to an increase in Pi content, these plants also exhibiting resistance to infection by *P. cucumerina* and *C. higginsianum*. Disease resistance is accompanied by increased ROS production. Furthermore, up-regulation of genes involved in salicylic acid (SA) and jasmonic acid (JA) signaling pathways occurs in Arabidopsis plants overaccumulating Pi, which is accompanied by a higher level of SA and JA in these plants. An opposite regulation of the two branches in the JA signaling pathway, the ERF1/PDF1.2 and MYC2/VSP2 branches, is observed in high Pi plants. While the ERF1-PDF1 branch positively responds to fungal infection in wild type plants and high Pi plants (miR399 overexpressor, *pho2* mutant plants), the MYC2/VSP2 branch is negatively regulated during infection in high Pi plants (but not in wild-type plants). Together, these findings support that miR399 plays a positive role in regulating hormone levels and immune responses in Arabidopsis.

Chapter II describes the impact of alterations in the expression of *MIR827* and its target gene *NLA* (NITROGEN LIMITATION ADAPTATION) in the response of Arabidopsis plants to infection by pathogenic fungi. Both miR827 overexpressing and *nla* mutant lines overaccumulated phosphate and were more resistant to *P. cucumerina* infection. The *nla* plants show higher callose deposition and ROS production, a phenomenon that is also observed in Arabidopsis plants grown under high Pi conditions. Besides, in the absence of pathogen infection, genes involved in the SA- and JA-signaling pathways are up-regulated in *nla* plants compared to wild type plants. Accumulation of SA and JA also occurs in *nla* mutant plants. Additionally, loss-of-function of *nla* results in accumulation

of the phytoalexin camalexin. These results support that *NLA*, the target gene of miR827 in Arabidopsis, negative regulates defense responses in Arabidopsis.

C**hapter III** describes the contribution of miR827 in the regulation of Pi homeostasis and resistance to pathogen infection in rice. For this investigation, CRISPR/Cas9-edited *miR827* plants were produced. These lines together with miR827 overexpressor plants were characterized and assayed for disease resistance against the blast fungus *Magnaporthe oryzae*. Compared with wild-type plants, the CRISPR/Cas9-edited miR827 plants and miR827 overexpressor rice plants showed lower and higher Pi content, respectively. While CRISPR/Cas9-mediated *MIR827* editing enhances resistance to infection by *M. oryzae*, miR827 overexpression increases disease susceptibility. Using ^{31}P-HR-MAS, differences in the vacuolar-to-cytosolic distribution of Pi were detected between loss-of-function and gain-of-function miR827 plants. These findings support the notion that, not only total Pi content, but also disturbance in the subcellular distribution of Pi (e.g. between vacuoles and cytoplasm) might have an impact on resistance to infection by the blast fungus *M. oryzae* in rice.

Resumen

En la naturaleza, las plantas se enfrentan constantemente a estreses abióticos y bióticos que afectan gravemente su productividad. Las investigaciones realizadas hasta la fecha con el objetivo de comprender la respuesta de las plantas a estos tipos de estreses se han realizado para cada estrés de forma individual. Una posible consecuencia de la exposición simultánea a varios tipos de estreses es que la respuesta de defensa de la planta a un estrés particular podría tener un efecto positivo o negativo en la respuesta a otro tipo de estrés debido a la interacción entre sus respectivas vías de señalización.

Durante el curso de la evolución, las plantas han desarrollado diferentes mecanismos que les permiten defenderse de la infección por patógenos. Actualmente, existe una gran cantidad de información en la literatura sobre la reprogramación transcripcional de la expresión génica en plantas durante la infección por patógenos. Diferentes estudios respaldan que la respuesta inmune de las plantas puede regularse postranscripcionalmente mediante la actividad de los microARNs (miARNs). Sin embargo, existe poca información disponible sobre los miARN que participan en la interacción entre las vías de señalización inducidas por patógenos y la señalización por nutrientes en las plantas. Por otro lado, se ha demostrado que el suministro de nutrientes (por ejemplo, la fertilización) tiene un impacto en la resistencia a la infección por patógenos en los ecosistemas agrícolas.

El fósforo es un nutriente esencial para el crecimiento y desarrollo de las plantas. Las plantas adquieren este nutriente del suelo en forma de fosfato inorgánico (Pi). La baja biodisponibilidad de Pi en suelos agrícolas representa un importante factor limitante para el crecimiento de las plantas. Como consecuencia, los fertilizantes basados en Pi se utilizan de forma rutinaria en la agricultura moderna para optimizar el rendimiento de los cultivos, lo que lleva a un escenario de exceso de Pi en muchos ecosistemas agrícolas. Al mismo tiempo, los plaguicidas se utilizan comúnmente para la protección de cultivos contra la infección por patógenos. El uso excesivo de fertilizantes y pesticidas tiene un impacto negativo en la salud humana y el medio ambiente. A nivel molecular, se ha

realizado un gran esfuerzo para comprender cómo las plantas se adaptan a las condiciones limitantes de Pi a través de la llamada "Phosphate Starvation Response" (PSR). Dos miARN, miR399 y miR827, han sido descritos por ser importantes en la regulación de la homeostasis del Pi y la adaptación a las condiciones Pi limitante en las plantas. Se dispone de menos información sobre los mecanismos de adaptación al exceso de Pi en las plantas.

El tema principal de esta tesis se centra en la investigación de la interacción que existe entre la homeostasis del Pi y la respuesta inmune en Arabidopsis (Capítulo I y Capítulo II) y el arroz (Capítulo III). Específicamente, en esta tesis doctoral se ha investigado la regulación de la homeostasis del fosfato y la resistencia a enfermedades mediada por miR399 y miR827.

El **Capítulo I** describe la función de miR399 y su gen diana *PHO2* (*PHOSPHATE2*) en la regulación de la homeostasis del fosfato y la resistencia a enfermedades en Arabidopsis. Los hongos patógenos utilizados fueron: *Plectosphaerella cucumerina* (necrótrofo) y *Colletotrichum higginsianum* (hemibiótrofo). Los resultados obtenidos en este estudio indicaron que el crecimiento de plantas en alto Pi, así como la sobreexpresión de miR399 y la pérdida de función de las plantas PHO2, conduce a un aumento en el contenido de Pi, exhibiendo estas plantas una mayor resistencia a la infección por *P. cucumerina* y *C. higginsianum*. La resistencia observada viene acompañada de una mayor producción de ROS. Además, se ha observado que las plantas de Arabidopsis que sobre acumulan Pi presentan una inducción de genes implicados en las vías de señalización del ácido salicílico (SA) y ácido jasmónico (JA), lo que se traduce en un mayor nivel de SA y JA en estas plantas. También se ha observado una regulación opuesta de las dos ramas de la vía de señalización del JA, las ramas ERF1 / PDF1.2 y MYC2 / VSP2, en plantas de alto contenido en Pi. Mientras que la rama ERF1-PDF1 responde positivamente a la infección por hongos en plantas de tipo silvestre y plantas con alto Pi (sobreexpresoras del miR399 y plantas mutantes pho2), la rama MYC2 / VSP2 está regulada negativamente durante la infección en plantas con alto Pi (pero no en plantas de tipo silvestre). Juntos, estos hallazgos respaldan que miR399 desempeña un papel positivo en la regulación de los niveles hormonales y la respuesta inmune en Arabidopsis.

El **Capítulo II** describe el impacto causado por la alteración de la expresión de MIR827 y su gen diana *NLA* (*NITROGEN LIMITATION ADAPTATION*) en la respuesta de las plantas de Arabidopsis a la infección por hongos patógenos. Tanto las líneas de sobreexpresión de miR827 como las mutantes *nla* sobreacumularon fosfato y fueron más resistentes a la infección por *P. cucumerina*. Las plantas *nla* muestran una mayor deposición de callosa y producción de ROS, un fenómeno que también se observa en plantas de Arabidopsis cultivadas en condiciones de Pi elevado. Además, en ausencia de infección, los genes implicados en las vías de señalización de SA y JA se regulan positivamente en las plantas *nla* en comparación con las plantas de tipo salvaje. La acumulación de SA y JA también se produce en plantas mutantes *nla*. Por otro lado, la pérdida de función de *NLA* da como resultado la acumulación de la fitoalexina camalexina. Estos resultados apoyan que NLA, el gen diana de miR827 en Arabidopsis, regula negativamente la respuesta de defensa en Arabidopsis.

El **Capítulo III** describe la contribución de miR827 en la regulación de la homeostasis del Pi y la resistencia a la infección por patógenos en arroz. Para llevar a cabo esta investigación, se obtuvieron plantas mutantes para miR827 editadas con la tecnología CRISPR / Cas9. Estas líneas, junto con plantas sobreexpresoras de miR827, se caracterizaron y ensayaron para determinar la resistencia a enfermedades frente al hongo *Magnaporthe oryzae*. En comparación con las plantas de tipo silvestre, las plantas miR827 mutadas por CRISPR / Cas9 y las plantas de arroz sobreexpresoras del miR827 mostraron un contenido de Pi más bajo y más alto, respectivamente. Mientras que la edición de MIR827 mediada por CRISPR / Cas9 mejora la resistencia a la infección por *M. oryzae*, la sobreexpresión de miR827 aumenta la susceptibilidad a la enfermedad. Usando la técnica de ^{31}P-HR-MAS, se detectaron diferencias en la distribución de Pi entre vacuola y citosol para las plantas miR827 mutantes y sobreexpresoras. Estos hallazgos apoyan la idea de que, no solo el contenido total de Pi, sino también la alteración en la distribución subcelular de Pi (por ejemplo, entre las vacuolas y el citoplasma) podría tener un impacto en la resistencia a la infección por el hongo *M. oryzae* en arroz.

General introduction

The General Introduction is a modified version of the review published in the Agricultural Journal

Authors: Beatriz Val-Torregrosa, Mireia Bundó, Blanca San Segundo

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1. *Arabidopsis thaliana*

1.1. *Arabidopsis thaliana*: Origin and morphological characteristics

Arabidopsis thaliana (thale cress or mouse-ear cress) is an herbaceous plant that belongs to the *Brassicaceae* family, which includes important crops like cabbage (*Brassica oleracea*), radish (*Raphanus sativus*) or the black mustard (*Brassica nigra*). *Arabidopsis thaliana* is a widely distributed annual selfing plant species found in a large range of environmental conditions.

A. thaliana has a short life cycle that can be as short as 6–8 weeks in some accessions. The plant has wide leaves at the base, forming a rosette, and a long stem than can reach 30 or 40 cm, where the raceme inflorescences can be found (**Figure 1A**). The inflorescences are organized in groups of small hermaphrodite white flowers that are usually self-pollinated. Once the flowers are pollinated, the ovary changes its morphology and becomes a fruit in the form of silique with the seeds inside (**Figure 1A**) (Meyerowitz, 1987).

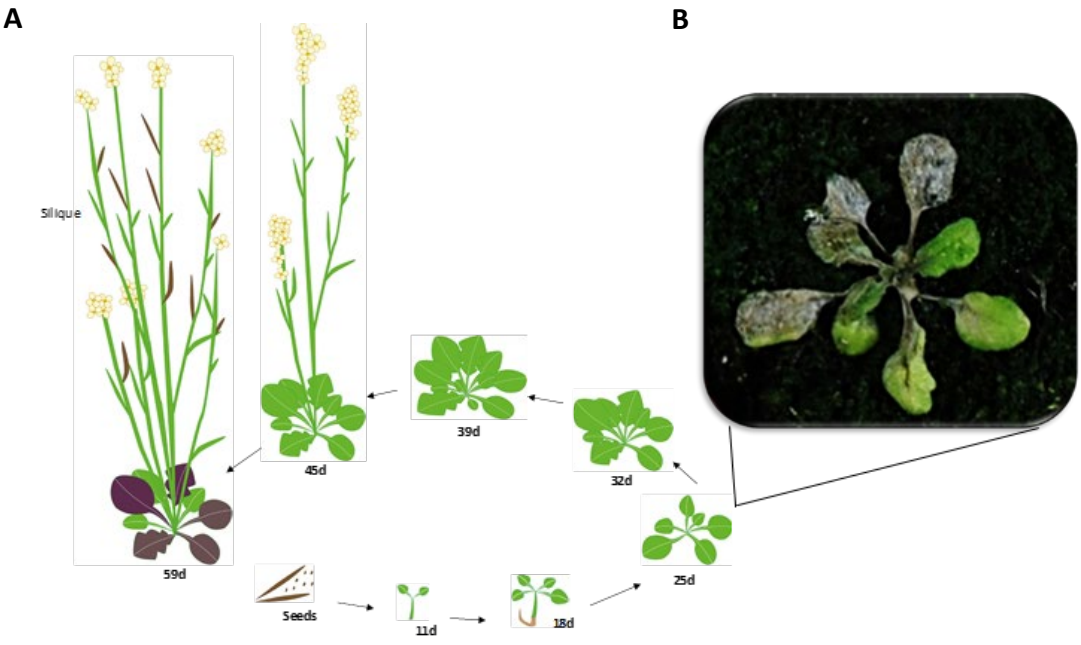

Figure 1. **A.** The Arabidopsis life cycle from seed to seedling, vegetative growth and reproductive stage. Once the seed germinates, the first seedling with two cotyledons appears. As the days go by, leaves increase in size and number. The leaves of *A. thaliana* are positioned to form a compact rosette, and inflorescences form a large number of siliques. Adapted from Krämer, 2015. **B.** Leaf symptoms of three weeks old *A. thaliana* plant infected with *Plectosphaerella cucumerina.*

Despite Arabidopsis is not a species with agronomic significance, it is very useful for basic research in genomics and molecular biology studies. *A. thaliana* emerged as the model species in functional genomic studies because of is its small genome (aprox 125 Mb) with only 5 chromosomes, and its short life cycle. *A. thaliana* was the first plant to have its genome sequenced (Kaul *et al.*, 2000). It is a small plant, thus giving the possibility of growing a high number of plants in a tiny space and in a short time. Moreover, large collections of mutant lines and genomic resources are available. Arabidopsis is amenable to Agrobacterium-mediated transient transformation as well as to the floral drip or dip transformation method. This makes Arabidopsis an easy plant to work with in research programs.

1.2. *Arabidopsis thaliana*: a model for the study of innate immunity in dicotyledonous plant species.

Different fungal and bacterial pathogens are being used for the study of defense responses in Arabidopsis. In the case of fungal pathogens, fungi with different life-styles have been described to infect *A. thaliana*, including obligate biotrophs (*Puccinia sp.*), hemibiotrophs (*Colletotrichum sp.*) and necrotrophs (*Botrytis sp.*, *Fusarium sp.*, *Plectosphaerella sp*.). (Jakob *et al.*, 2002; Dean *et al.*, 2012). Regarding bacterial pathogens, the most widely used is *Pseudomonas syringae,* followed by *Ralstonia solanacearum* (a high destructive bacterial pathogen for several plant species) (Deslandes *et al.*, 2002; Genin, 2010; Balagué *et al.*, 2017; Velásquez *et al.*, 2017).

The Arabidopsis/*Pseudomonas syringae* pathosystem is one of the best characterized plant/pathogen interactions (Katagiri *et al*., 2002; Lyons *et al.*, 2015). *P. syringae* enters into the host through wounds or natural openings in the surface of the plant and once inside it proliferates, being able to kill the plant.

1.2.1. *Plectosphaerella cucumerina*

Plectosphaerella cucumerina (previously known as *Fusarium tabacinum*; anamorph *Plectosporium tabacinum*) (Palm *et al*., 1995) is an ascomycete fungus with a necrotrophic life style that causes sudden death and blight disease. The fungus grows and proliferates on damaged plant tissue causing a severe disease, ending with the host

death. *P. cucumerina* is a soil-born fungus that affects a high number of crops, like pumpkin or snap beans, but also weed-like plants as Arabidopsis (Ton and Mauch-Mani, 2004; Dillard *et al.*, 2005; Sato *et al.*, 2005; Ramos *et al.*, 2013). The typical symptoms in Arabidopsis include leaves with brown spots lesions that spread throughout the plant in a short period of time (**Figure 1B**) (Berrocal-Lobo *et al*., 2002). The Arabidopsis/*P. cucumerina* pathosystem has been adopted as a model for studies of the plant immune response against necrotrophic fungal pathogens.

1.2.2. *Colletotrichum higginsianum*

Colletotrichum spp. is an ascomycete fungus that embraces different species that are able to infect many different plant species worldwide. This fungus triggers anthracnose and blight symptoms causing important crop losses (Dean *et al.*, 2012). Among them, *Colletotrichum higginsianum* affects cruciferous species like *Brassica* or Arabidopsis. *C. higginsianum* is a hemibiotrophic fungus with a short biotrophic phase in which it develops infective structures like appressoria and biotrophic hyphae. Then, it switches to a second necrotrophic phase, triggering the plant's death. The easy culture of this fungus in axenic media, the existence of T-DNA transformation protocols and the availability of its complete genome, makes the pathosystem *A. thaliana*/ *C. higginsianum* an important model to study plant-fungal interactions. (Yan *et al.*, 2018).

Another pathogen that infects *A. thaliana* is the fungus *Fusarium oxysporum*, a hemibiotrophic fungus that infects roots causing wilt disease (Berrocal-Lobo and Molina, 2008).

2. Rice

2.1. Rice cultivation: origin and economic importance

Rice (*Oryza sativa* L.) is a monocotyledonous plant that belongs to the *Poaceae* family. It is considered one of the most important crops in the world and the staple food of more than half of the global population. Morphologically speaking, rice has long leaves with a central vein connected to the stem. Rice inflorescences appear in the form of panicles containing the seeds (**Figure 2**, left panel).

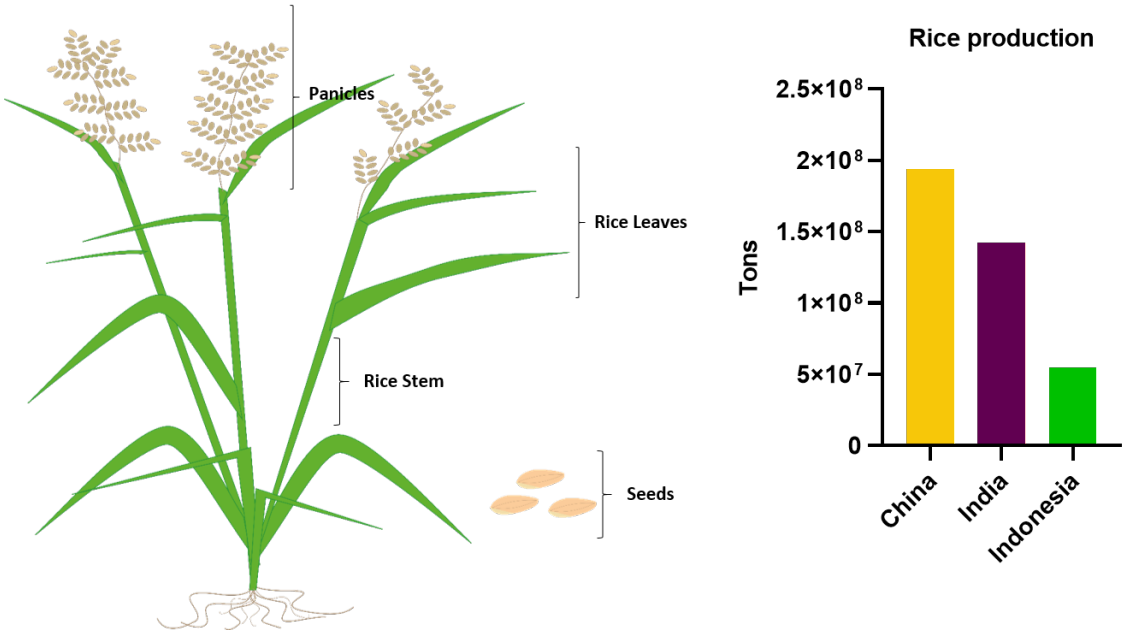

Figure 2: Left panel; illustration of an adult rice plant, representing the leaves shape and the appearance of the panicles containing the seeds. Right panel; Rice tons production by the three main producer countries (source FAOSTAT Database, 2019)

Rice, which originated around 9000 years ago in the Yangtze Valley (China), has a long history of natural selection and domestication (Gutaker *et al.*, 2020). The genus *Oryza* comprises 23 different species. Of them, only two species are cultivated, *Oryza sativa* and *Oryza glaberrima*, which were independently domesticated in Asia and Africa, respectively. *O. sativa* includes two important subspecies, *Oryza sativa spp. japonica* (with tropical and temperate subgroups) and *Oryza sativa spp. indica* (Sweeney and McCouch, 2007; Huang *et al.*, 2012; Gross and Zhao, 2014; Wang *et al.*, 2014). *Oryza rufipogon* and *Oryza nivara*, the two closest relatives of *O. sativa,* are considered to be the progenitors of the Asian cultivated rice, whereas *O. glaberrima* originated from its wild ancestor *Oryza barthii* (Linares, 2002; Londo *et al.*, 2006).

In addition to its agricultural relevance, rice represents the model plant for functional genomics research in monocotyledonous species. *O. sativa* was the first crop species to have its genome sequenced, and reference genomes are available for *japonica* and *indica* subspecies (Goff *et al.*, 2002; Yu *et al.*, 2002). Among cereals, rice has the smallest genome with an estimated size of 430 mega base pairs. More recently, the 3K Rice

Genome Project sequenced more than 3000 rice accessions which provided a tremendous resource for rice research (Li *et al.*, 2014).

China is the largest rice producer in the world, followed by India, Indonesia and Bangladesh (www.fao.org/faostat) (**Figure 2**, right panel). Rice is grown in a wide range of environments characterized by different temperatures, climates and irrigation conditions (paddy fields, seasonal flooding and upland rainfed conditions). To meet the forecasted demand of rice under the rapid increase in human population, rice production must significantly increase, and this should be achieved with less arable land due to urbanization, often polluted by indiscriminate use of agrochemicals and industrialization. Other important factors that rice farmers have to face are climate change and water scarcity. As rice is a water-demanding crop, water usage in rice production has to be optimized in order to maintain or even increase the productivity in this new scenario.

Rice is prone to various types of stresses, both biotic and abiotic. Biotic stresses, such as those caused by fungi, bacteria, viruses, insect pests or nematodes, represent a serious threat to sustainable rice production. To reduce losses due to pathogen infections and to obtain maximum yields, high rates of pesticides and fertilizers continue to be applied in rice farming, causing an adverse impact on human health and the environment. Furthermore, climate change is promoting the northerly movement of pests, which means that temperate rice agriculture should be concerned about tropical pests and diseases.

Abiotic stresses, such as drought, salinity, cold, high temperature, submergence, nutrient deficiencies, and heavy metal toxicity are also factors negatively affecting rice growth and productivity worldwide. As most of the global rice supply originates from flooded ecosystems, the rice crop has unique features in terms of adaptation to environmental stresses. Due to its high requirement of water, rice is one of the most affected crops by drought stress. Progress in agricultural research is needed for the development of management strategies that can capitalize on enormous variety of rice production systems in very different climates. Sustaining rice production under changing climate is a big challenge in coming years. To overcome problems caused by biotic and

abiotic stresses, new rice varieties with higher yield and improved tolerances need to be developed, taking advantage of all the resources and technologies currently available.

2.2. Rice pathogens

One of the most important pathogens affecting rice is the fungus *Magnaporthe oryzae,* the causal agent of the blast disease, which can infect leaves, leaf collars, necks and panicles of rice plants. *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 (Wilson and Talbot, 2009; Fernandez and Orth, 2018). The blast disease cycle starts when a conidium reaches the host tissue and develops the germ tube, which grows on the leaf surface before differentiating into the dome-shaped appressorium (**Figure 3**). A penetration peg develops from the appressorium to enter into the epidermal cells, where it expands to become an infective hypha. During the biotrophic stage, hyphae moves 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 (**Figure 3**) (Wilson *et al.*, 2012; Fernandez and Orth, 2018). Several days after infection, the fungus switches to a necrotrophic life style and blast lesions appear on the leaf surface, typically diamond-shaped with a gray or white center and brown or reddish brown border (**Figure 3**). The fungus sporulates profusely in these lesions, thus, allowing the disease to spread rapidly to adjacent rice plants. Neck rot and panicle blast are particularly devastating diseases that cause important yield losses in severe epidemics. Furthermore, *M. oryzae* is capable of infecting different crops apart from rice, like barley or wheat, which highlights its incredible ability to infect monocotyledonous plant species. The *M. oryzae* genome has been sequenced, and functional genomic approaches have led to the identification of hundreds of genes involved in its pathogenesis. Therefore, *M. oryzae* has become a model fungus for studying host-pathogen interactions, at both the cell biology and molecular level (Dean *et al.*, 2012)**.**

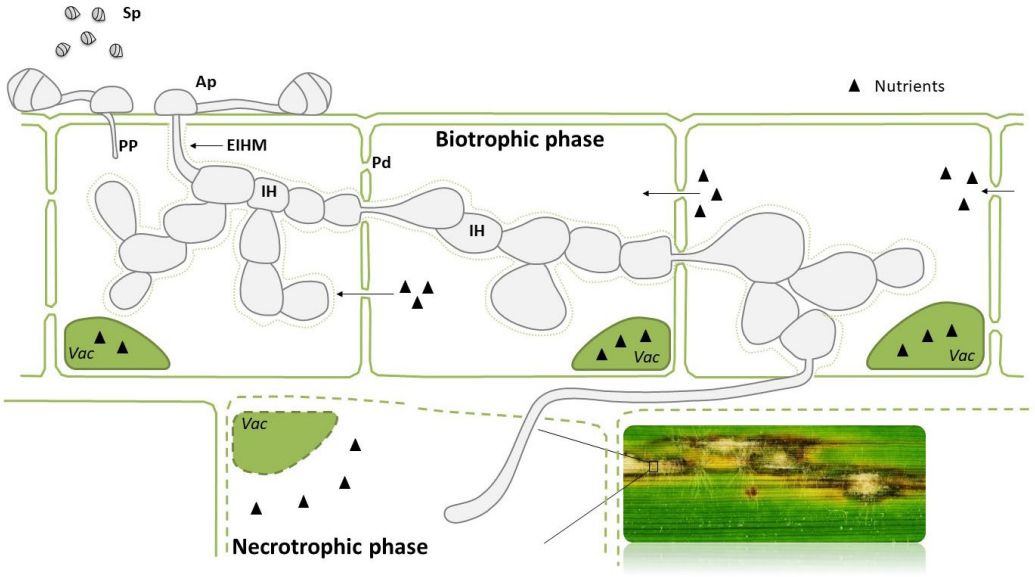

Figure 3: Colonization of rice leaves by the hemibiotroph fungus *Magnaporthe oryzae*. The fungus first establishes a biotrophic interaction with the rice plant and later switches to a necrotrophic lifestyle. During the biotrophic phase, the fungus grows inside living host cells. The hyphae are encased by the so called extra-invasive hyphal membrane (EIHM) which is continuous with the plant plasma membrane. At this stage of the infection process, the fungus uses plasmodesmata for cell-to-cell movement (Kankanala *et al.*, 2007; Sakulkoo *et al.*, 2018), and absorb nutrients from the plant living cells. The host vacuolar integrity in the first invaded rice cells is important for *M. oryzae* early stages of infection (Mochizuki, Minami and Nishizawa, 2015). In the necrotrophic phase, the fungus colonizes the host tissue, and obtains nutrients from the host dead cells. Ap, appresorium; EIHM, extra invasive hyphal membrane; IH, invasive hyphae; Pd, plasmodesmata; PP, penetration peg; Sp, spores; Vac, Vacuole.

Rice blast is a classical gene-for-gene interaction system, and numerous blast resistance (*R*) genes are identified in rice (Monteiro and Nishimura, 2018; Li *et al.*, 2019) . Also, numerous avirulence (AVR) genes have been genetically and functionally described in *M. oryzae* (Wang *et al.*, 2017). However, traditional resistance conferred by *R* genes often breaks down in a few years due to the high variability and fast evolving fungal populations. Therefore, breeders usually prefer rice varieties that contain multiple *R* genes and have broad-spectrum blast resistance (Wu *et al.*, 2019) 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 (Iwai *et al.*, 2007; Moselhy *et al.*, 2016). The wide use of these chemicals might, however, provoke the development of resistance in the pathogen and/or cause side effects on non-target organisms and the environment. 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.

Bakanae ("foolish seedling" in Japanese), caused by one or more seed-borne *Fusarium* species, mainly *F. fujikuroi*, is a disease of increasing economic importance. This fungus infects rice plants from the seedling stage to the mature stage, with severe infection of rice seeds (Iqbal *et al.*, 2011). *Rhizoctonia solani* is a necrotrophic soil fungus that infects rice plants by vegetative mycelia and sclerotia, causing the rice sheath blight (Raj *et al.*, 2019). Bacterial blight and bacterial leaf streak, caused by *Xanthomonas oryzae pv. oryzae* and *X. oryzae pv oryzicola*, respectively, are considered as major bacterial diseases in rice (Niño-Liu *et al*., 2006).

Rice is also vulnerable to a number of diseases caused by *viruses* which have become increasingly important around rice growing areas in the tropics (Geering and Randles, 2012), e.g., Rice hoja blanca virus (RHBH) in South-America and Rice Yellow Mottle Virus (RYMV) in Africa. Rice stripe virus (RSV) and Rice tungro viruses (RTVB and RTSV) are found in Asia. Most of the rice viruses are transmitted by insect vectors through persistent or non-persistent manner.

Insect pests are also important biological constraints limiting rice yield. Stem borers, in particular the striped stemborer (SSB) (*Chilo suppressalis*), is a major constrain on rice production. The insecticidal Cry proteins produced by *Bacillus thuringiensis,* and rice varieties expressing the BT cry-endotoxin genes, have provided some tools for the control of a wide diversity of insect pests (Breitler *et al.*, 2001).

Clearly, integrative strategies are required for successful control of rice diseases, including the use of advanced breeding lines and cultivars, forecasting distribution of the disease and pathogen races, examining wild *Oryza* species for novel sources of resistance, as well as using appropriate disease and water management systems. Modern biotechnology offers new opportunities for the development of rice varieties with enhanced disease resistance, which will be even more crucial if rice yields are going to be increased using sustainable strategies.

3. Simultaneous biotic and abiotic stresses

In nature, plants are simultaneously exposed to combinations of stress conditions, including simultaneous biotic and abiotic stresses. However, most studies to determine the effects of environmental stress have been performed on plants exposed to an individual stress, and few research efforts focused on the impact of combined abiotic and biotic stresses in plants. It is also known that cross-talk between plant responses induced by one stress may result in positive or negative impact over the other stress (Saijo and Loo, 2020). As an example, drought stress increases susceptibility to the rice blast fungus (Bidzinski *et al.*, 2016). Here, drought stress severely reduces basal immunity and provokes the partial breakdown of several major resistance genes, affecting the fungal virulence program.

A priori, the occurrence of abiotic stresses might alter plant–pathogen interactions by altering plant physiology and/or defense responses. Regarding the impact of nutrient stress on disease resistance, the outcome of the interaction is difficult to predict, as different results have been observed depending on the host plant, the type of pathogen and the fertilization treatment. At present, fertilizers containing plant nutrients are commonly used to maintain optimal yield in crops cultivation. Although increasing rice yields, the indiscriminate use of fertilizers might have adverse effects on the plant by increasing the likelihood of disease. It has long been recognized that excess of nitrogen fertilization increases susceptibility to infection with the blast fungus *M. oryzae* (Ballini *et al*., 2013). More recently, it was reported that high phosphate fertilization also enhances susceptibility to *M. oryzae* in rice plants (Campos-Soriano *et al*., 2020). There is the need to develop eco-friendly technologies to maintain productivity while reducing inputs of agrochemicals, fertilizers and pesticides, also in response to legislative and public pressure. To fully understand the impact of nutrient stress in disease resistance, it is important to investigate the nature of such interactions.

4. Nutrient stress in plants

Nutrients, macronutrients (nitrogen, phosphorus, potassium) and micronutrients (iron, copper, zinc, manganese, nickel…) (Satpathy *et al*., 2014) are taken from the soil and

play a very important role in plant growth and development. Nevertheless, nutrient stress can be a major cause of yield reduction. Nutrient stress refers to deficiencies of essential plant nutrients as well as excess of nutrients causing toxicity. Under nutrient stress conditions, the plant must respond dynamically to maintain nutrient levels in the optimal range for normal growth and development. Physiological disorders, also called physiological diseases, are quite common in rice grown under poor soil conditions (Tahir *et al.*, 2019). Contaminated soils, irrigation sources and agrochemicals contribute to heavy metal and micronutrient stress in crop plants. This affects the growth and development of majority of plant species and significantly reduces the yield (Satpathy *et al*., 2014).

4.1. Plant responses to nutritional stress

Plants naturally evolved physiological mechanisms and adaptation strategies to cope with nutrient stresses. Under limiting nutrient conditions, plants change the root system architecture by stimulating root branching and can secrete small molecules into the soil to enhance the nutrient's bioavailability. Nutrient limitation might also lead to induction of genes encoding nutrient transporters to increase uptake, and genes involved in recycling or reallocation of nutrients to maintain nutrient levels in the optimal range during growth and development. Under nutrient excess, the plant responds through modification of nutrient acquisition, detoxification pathways, and sequestration into cellular compartments (e.g. vacuoles).

Nitrogen (N) is a component of important molecules, like amino acids, proteins or nucleic acids. The most available forms of nitrogen in the soil are nitrate and ammonium. Plants have developed different strategies to overcome low N in the soil, aiming to enhance N use efficiency through the regulation of N utilization and acquisition. Alterations in root architecture and increased activity of influx transporters are the major determinants to increase N acquisition efficiency (Kiba and Krapp, 2016).

Phosphorous (P), like N, is a main macronutrient in plants. It is a component of nucleic acids and phospholipids, and plays crucial roles in enzymatic reactions and signal transduction cascades (e.g. protein phosphorylation). P is also involved in energy transfer mechanism (ATP). In plants, phosphorous is absorbed through the roots in the

form of inorganic phosphate (Pi). Even though the overall content of Pi in the soil is generally high, its low bioavailability represents a limiting factor for plant growth in many agricultural ecosystems (Hinsinger *et al.*, 2011). P deficiency is widespread in major rice ecosystems and is the major growth-limiting factor in acid upland soils where soil P-fixation capacity is often important. To explore the soil, the Pi-starved plants change their root architecture by increasing lateral roots and root hairs (Huang and Zhang, 2020). The most evident symptoms of Pi deficiency in plants are the reduction of plant growth and development of thinner leaves with darker appearance (Malhotra *et al*., 2018). Pi-deprived plants experience important transcriptional reprogramming aiming to increase Pi acquisition in the root, translocation from the root to the shoot, and mobilization of Pi stored in the vacuole, the so called **Phosphate Starvation Response** (PSR) (Wang *et al*., 2021; Puga *et al.*, 2017; Chien *et al.*, 2018). Another strategy developed by Pi-starved plants is an increase of RNAses and phosphatase activities, these enzymes being involved in metabolic Pi recycling (Tadano and Sakai, 2012; Gho *et al*., 2020). Pi excess in the soil also has an effect on root development, as the plants show a reduction in primary root growth and less meristematic activity (Shukla *et al*., 2017). Excessive Pi accumulation leads to toxicity on the tip of rice leaves (leaf tip necrosis) which is more evident in older leaves, as also occurs in Arabidopsis.

Potassium (K) is another important inorganic nutrient for plant growth and production. It plays important roles in plant cell processes related with stomata closure, photosynthesis, turgor pressure, ion transport or enzyme reactions as a cofactor. Although most soils are rich in K minerals, relatively little K is present in forms that are available to plants. When the plant senses the lack of this nutrient in the soil, it triggers the activation of channels and K transporters, which in some cases is accompanied by the induction of other cellular processes that are also regulated by Ca^{2+}, ROS and hormones such as abscisic acid (ABA) (Srivastava *et al*., 2020).

Although required in smaller amounts than macronutrients (N, P, K), micronutrients are also essential for plant growth and play an important role in balanced crop nutrition. Iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), molybdenum (Mo) and nickel (Ni) are metal micronutrients that function in a range of cell's chemical reactions. Silicon (Si) and

boron (B) mainly contribute to the plant's structure, while the major role of the non-metal chlorine (Cl) serves in osmotic balance.

Finally, plants are also susceptible to heavy metals contamination such as cadmium (Cd) and arsenic (As) coming from contaminated soil, river or groundwater. Upon exposure to heavy metals, plants experience oxidative stress that leads to cellular damage and disturbance of cellular ionic homeostasis. These toxic metals accumulate in rice grains and are significant risks for human and animal health. Water management practices significantly influences the Cd and As concentration in rice grains (Rizwan *et al*., 2016).

4.2. The phosphate starvation response

Due to the low availability of phosphate in the soil, plants had to develop different strategies to increase Pi content. Thus, under Pi limiting conditions, plants activate the so called Phosphate Starvation Response (PSR) (Yang and Finnegan, 2010; Chien *et al.*, 2018). Most of our knowledge on this issue comes from studies in the model plant *A. thaliana* where Pi starvation responses are mainly regulated by the transcription factor PHOSPHATE REGULATOR 1 (*AtPHR1*) (*OsPHR2* is the orthologous gene in rice) (Panigrahy *et al*., 2009; Bustos *et al.*, 2010). These PHR transcription factors regulate the expression of the PSR components by recognizing their *PHR1-binding sequences* (P1BS) *cis*-regulatory elements, normally located in the 5' UTR (Bustos *et al.*, 2010).

The implication of miR399 in the PSR is well documented (Fujii *et al.*, 2005; Chiou *et al.*, 2006; Hsieh *et al.*, 2009; Puga *et al.*, 2017; Ham *et al.*, 2018).When the plant sense the lack of Pi, PHR1 induces the expression of *MIR399* in roots which, in turn, down-regulates the expression of *PHO2* (PHOSPHATE 2). *PHO2* encodes an ubiquitin E2 conjugating enzyme that mediates degradation of Pi transporters. In this way, miR399 accumulation relieves negative post-transcriptional control of PHT1 family Pi transporters for an increase in Pi uptake. The PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (*PHF1*) helps with the translocation of PHT1 transporters from the endoplasmic reticulum to the plasma membrane (Liu *et al.*, 2014). On the other hand, *MIR399* displays strong induction in shoots during Pi limitation (Bari *et al.*, 2006). Thus, micro-grafting experiments using Arabidopsis shown that chimeric plants constitutively over-expressing miR399 in the shoot accumulated mature miR399 species to very high

levels in their wild-type roots, while corresponding primary transcripts are virtually absent in roots. miR399 movement from shoots to roots serves as a long-distance signaling molecule in regulating Pi homeostasis during Pi deprivation at the whole-plant level (Fujii *et al.*, 2005; Aung, 2006; Chiou, 2006). The activity of miR399 is also regulated by INDUCED BY PHOSPHATE STARVATION (*AtIPS1*), a transcript that works kidnapping miR399 by target mimicry (Franco-Zorrilla *et al.*, 2007). The miR399/PHO2 module has a conserved function in controlling phosphate homeostasis in Arabidopsis and rice plants (**Figure 4**). *PHO2* also directs the protein degradation of PHO1 (PHOSPHATE1) which has been implicated in Pi loading to xylem for root-to-shoot Pi translocation (Liu *et al.*, 2012).

MiR827 is another miRNA involved in PSR, and its expression is controlled by *AtPHR1/OsPHR2* in Arabidopsis and rice, respectively (**Figure 4**). miR827 appears to function via different pathways in Arabidopsis and rice. In Arabidopsis, miR827 targets NITROGEN LIMITATION ADAPTATION (*AtNLA*) encoding an ubiquitin E3 ligase that mediates degradation of the plasma membrane-localized Pi transporter PHT1 (**Figure 4**, left panel) (Lin *et al*., 2013; Park *et al*., 2014). In this way, in Arabidopsis under Pi starvation conditions, miR399 and miR827 mediate cleavage of *AtPHO2* and *AtNLA* transcripts, respectively, resulting in PHO1 and PHT1 protein accumulation and increased Pi uptake and root-to-shoot allocation (**Figure 4**, **l**eft panel) (Liu *et al.*, 2014). In rice, miR827 targets two different *OsSPX-MFS* genes (*OsSPX-MFS1* and *OsSPX-MFS2*) (**Figure 4**, right panel). The SPX domain (named after proteins SYG1 ⁄PHO81 ⁄ XPR1) is found in several Pi transporters or signalling proteins in plants (Secco *et al.*, 2012). Their orthologue in Arabidopsis, the *AtPHT5* gene, was already characterized to be an influx Pi transporter into the vacuole (Liu *et al.*, 2016). Similarities between *OsSPX-MFS1*, *OsSPX-MFS1* and *AtPHT5*, and their common localization at the rice tonoplast, makes them candidates to be vacuolar phosphate transporters (Lin *et al.*, 2010, 2018). Thus, it has been proposed that in rice, miR827 functions in Pi compartmentalization and Pi storage (Lin *et al.*, 2010, 2018; Wang *et al.*, 2012; Liu *et al.*, 2016).

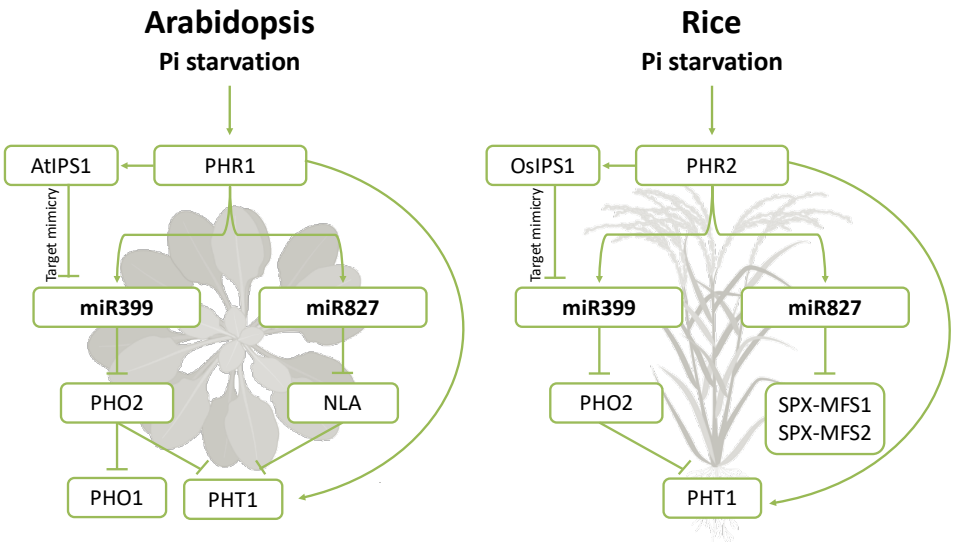

Figure 4: Arabidopsis and rice phosphate starvation response (PSR) pathways. Under phosphate starvation conditions the Arabidopsis transcription factor *AtPHR1* (*OsPHR2* in rice) activates *MIR399* and *MIR827* expression. In Arabidopsis and rice, *miR399* accumulation leads to degradation of *PHO2* transcripts resulting in the accumulation of phosphate transporters (PHT1 family). In Arabidopsis, *AtNLA* transcripts are cleaved by miR827, whereas rice miR827 targets two vacuolar Pi transporters, Os*SPX-MFS1* and *OsSPX-MFS2*.

5. Plant innate immunity

Plants have evolved multiple mechanisms to perceive and respond to pathogen infection. Resistance to pathogen infection relies on numerous changes at the biochemical, physiological and molecular level, largely coordinated by a massive and fast reprogramming of the host transcriptome. Upon contact with the plant, conserved molecular signatures derived from microbes, known as pathogen-associated molecular patterns (PAMPs; or microbe-associated molecular patterns, MAMPs) are recognized by Pattern Recognition Receptors (PRRs) localized in the plasma membrane (Jones and Dangl, 2006; Boller and Felix, 2009; Cui *et al*., 2015; Couto and Zipfel, 2016; Saijo and Loo, 2020). Examples of MAMPs include bacterial flagellin, lipopolysaccharide, EF-Tu, lipoproteins, peptidoglycans, and fungal chitin (Choi and Klessig, 2016). Plant PRRs fall into the receptor-like kinase (RLK) or receptor-like protein (RLP) families (Tang *et al*., 2017). PAMP recognition triggers the activation of a general defense response referred to as PAMP-triggered immunity (PTI) in which multiple signal transduction pathways operate. Plants can also detect their own molecules such as oligogalacturonides

released from plant cell walls as a consequence of physical damage caused by the pathogen infection, referred to as Damage-Associated Molecular Patterns (DAMPs), that are also able to initiate innate immune responses (Ferrari *et al*., 2013; Choi and Klessig, 2016). Extracellular ATP and endogenous elicitor peptides can also function as danger signals for the activation of the innate plant immune response (Tanaka *et al.*, 2014a). Downstream molecular events triggered by PAMP recognition includes the production of reactive oxygen species (ROS), reinforcement of the cell wall and formation of cell wall appositions (papillae), activation of phosphorylation/dephosphorylation cascades, and production of antimicrobial compounds (Yu *et al.*, 2017; Bacete *et al.*, 2018; Peng *et al.*, 2018; Lee *et al.*, 2020).

Some pathogens are able to produce effector proteins that are delivered into de host cell to suppress PTI responses. Therefore, the pathogens carrying these effectors are able to cause diseases on their host plants (Cui *et al*., 2015). During plant–pathogen coevolution, however, plants have developed resistance (R) proteins, encoded by resistance (*R*) genes, that recognize microbial effectors (or host proteins modified by effectors). This recognition triggers a rapid and robust host defense response, known as Effector-triggered immunity (ETI) (Jones and Dangl, 2006; Cui *et al*., 2015). Most R proteins belong to the nucleotide-binding site (NB)/leucine-rich-repeat (LRR) receptor family (NLR) (Monteiro and Nishimura, 2018). In plants, it is possible to find different classes of R-proteins, the toll-interleukin 1 receptors (TIR) characteristic from dicotyledonous, and the CNLs with a coiled-coil (CC) domain in monocotyledonous and dicotyledonous (Yue *et al.*, 2012; Jacob *et al*., 2013). The activity regulation of these proteins is controlled by conformational changes, allowing the N-terminal domains to interact with the effectors (Takken and Goverse, 2012). ETI is often accompanied by a form of programmed cell death at the site of pathogen infection, the hypersensitive response (HR), that restricts the spread of infection by microbial pathogens. Accumulating evidence supports that PTI and ETI share a number of signaling components and induce qualitatively similar transcriptional changes (Lu and Tsuda, 2021). These responses are, however, associated with quantitatively and kinetically different transcriptional changes in the host (**Figure 5**) (Peng *et al*., 2018).

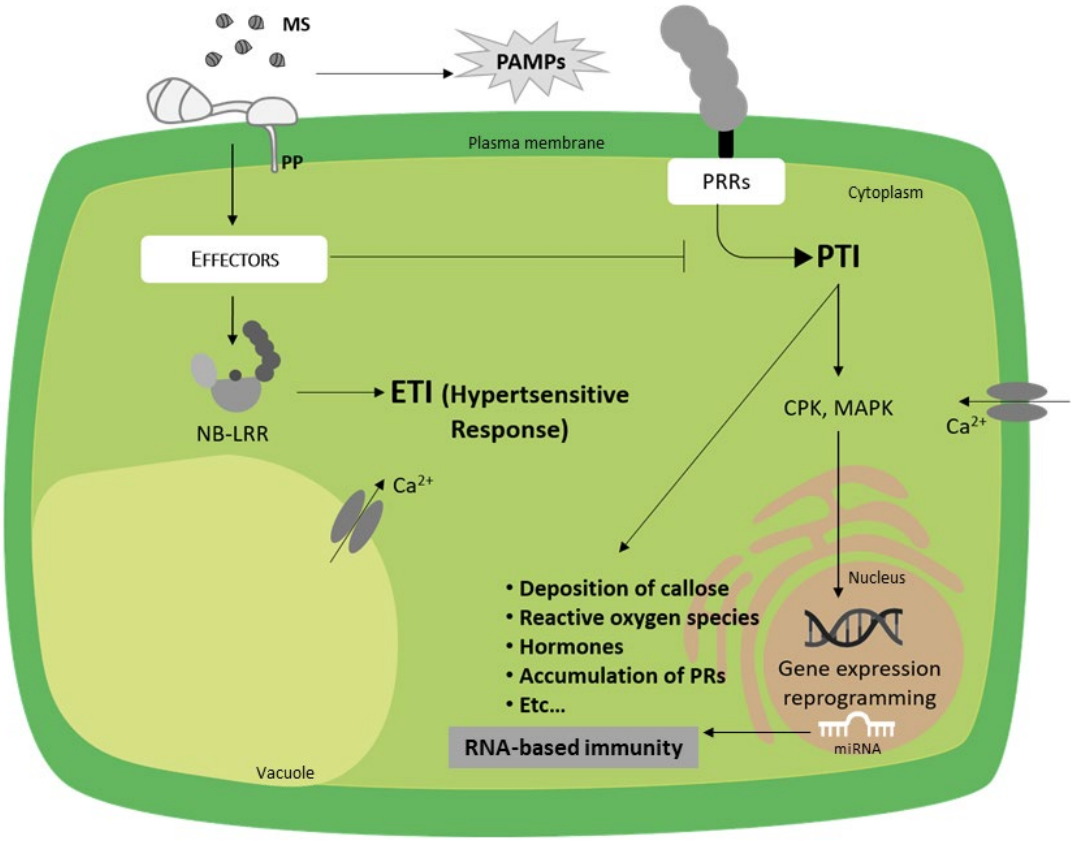

Figure 5: Schematic representation of signaling pathways in the plant response to pathogen infection. Typical responses associated to PTI (PAMP-triggered immunity) and ETI (Effector-triggered immunity), as well as the RNA-based immune system are represented. PAMPS, pathogen associated molecular patterns; PRR, pattern recognition receptor; MPKs, mitogen-activated protein kinases; CDPKs, calcium-dependent protein kinases; MS, *Magnaporthe oryzae* spore; NB-LRR, Nucleotide binding domain-leucine rich repeat R protein; PP, Penetration peg.

One of the early events in the plant response to pathogen infection is the increase in cytoplasmic Ca^{2+} which, in turn, activates phosphorylation cascades mediated by calcium-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs) (Seybold *et al.*, 2014). Pathogen-induced defense responses include the production of ROS, such as hydrogen peroxide (H_2O_2), superoxide radicals (O^{2-}) and hydroxyl radicals (^{-}OH) (Jones and Dangl, 2006). ROS are reactive molecules with multiple functions in plant defense reactions to pathogen infection. In particular, H_2O_2 possess antimicrobial activity and contributes to cell-wall reinforcement (e.g. lignification and oxidative cross-linking of cell-wall polymers) (Wang *et al.*, 2021). ROS also function as signaling molecules for the induction of other defense responses. Another free radical that is produced in the plant during infection is nitric oxide (NO).

NO seems to work together with ROS radicals in triggering cell death upon pathogen challenge and is also strongly related with Ca^{2+} and ABA signaling for regulation of the stomata closure (Van Meeteren *et al.*, 2020).

Plants also produce proteins and secondary metabolites that can be toxic to phytopathogens. Perhaps the best-known examples of defensive metabolites are phytoalexins, which are synthesized *de novo* in response to infection. *Brassicaceae* plant species accumulate camalexin, a tryptophan-derived phytoalexin with antifungal and antibacterial activity against phytopathogens (He *et al.*, 2019). Indol glucosinolates are also effective to limit colonization by necrotrophic fungi in plants (Sanchez-Vallet *et al.*, 2010). The accumulation of Pathogenesis-Related (PR) proteins is also a ubiquitous response of plants to pathogen infection, some of these proteins exhibiting antimicrobial activity (Van Loon and Van Strien, 1999).

Hormones play a crucial role in disease resistance by regulating plant immune responses (Aerts *et al*., 2021). Immune responses are coordinated mainly by the phytohormones salicylic acid (SA), ethylene (ET), jasmonic acid (JA and JA-derivatives), as well as by auxins and brassinosteroids. Plant hormones do not function independently, as synergistic and antagonistic interactions between hormone signaling pathways ultimately drive the fine-tuning of plant defense responses. Generally, the JA signaling pathway synergistically cross-talks with the ET signaling pathway, whereas the SA- and JA-pathways show negative crosstalk (Aerts *et al*., 2021). Hormone signaling networks in plant defense appear to be dependent on both the lifestyle of the pathogen in the host plant and the host plant itself. Whereas SA has been generally associated with plant defense against biotrophic pathogens, JA and ET are associated with protection against necrotrophic pathogens.

In Arabidopsis, the JA signaling pathway has two branches. On the one hand, the MYC2 branch is regulated by *AtMYC2* (a basic helix-loop-helix-leucine zipper transcription factor) (Yang *et al.*, 2019). *VSP2* (*Vegetative Storage Protein 2*) is routinely used as a marker gene of the MYC2 pathway which appears to be crucial in the plant response to herbivory attack (Aerts *et al*., 2021) . On the other hand, the *ERF1* branch, is regulated by *AtERF1* (APETALA/ERF transcription factor family). The activation of the ERF1 branch

is marked by the induction of *PDF1.2* (*Plant Defensin 1.2*) expression during pathogen infection (Lorenzo *et al.*, 2004; Pieterse *et al.*, 2012; Wasternack and Hause, 2013; Zhang *et al.*, 2017). A co-repression between ERF and MYC branches has been documented (**Figure 6**) (Lorenzo *et al.*, 2004; Wasternack and Hause, 2013; Aerts *et al*., 2021).

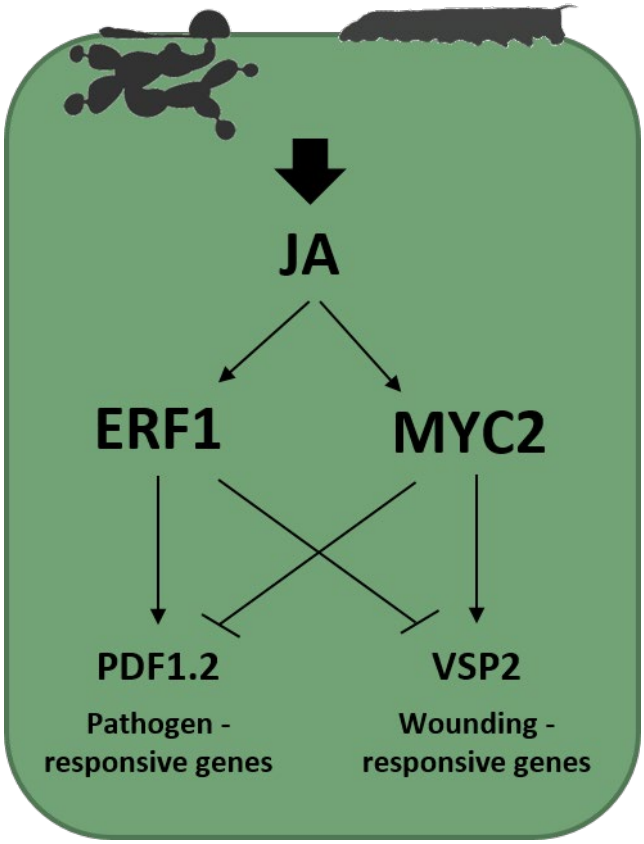

Figure 6: Representation of components of the JA signaling pathway composed of two different branches, the ERF2 and MYC2 branch, which are typically associated to the plant response to pathogen infection (ERF1/PDF1.2 branch) and herbivore attack (MYC2/VSP2) The ERF1 and MYC2 pathways mutually repress each other. Adapted from Lorenzo *et al.*, 2004.

Historically, plant immune responses to bacterial and fungal pathogens have been considered to relay on the function of protein-coding genes, whereas RNA-based mechanisms largely regulate plant-virus interactions. Along with this, the transcriptional control of protein-coding defense genes has been studied extensively, but less attention has been paid to the post-transcriptional regulation of defense-related processes. Nowadays, the contribution of host endogenous small RNAs (sRNAs) in the regulation of plant defense responses to infection by fungal and bacterial pathogens is well

demonstrated (Seo *et al.*, 2013; Staiger *et al.*, 2013; Islam *et al.*, 2018; Bundó *et al.*, 2020).

sRNAs are non-coding RNAs (20-24 nucleotides in length) involved in regulation of gene expression by transcriptional or post-transcriptional gene silencing (Baulcombe, 2004; Chapman and Carrington, 2007). In eukaryotes, there are two main classes of sRNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are distinguished by their mode of biogenesis, function and mechanisms of action.

6. microRNAS (miRNAs)

6.1. Biogenesis and mode of action of plant miRNAs

miRNAs are small non-coding RNAs (20-24 nucleotides) involved in post-transcriptional gene silencing through degradation or translational repression of their target transcripts, which are recognized in a sequence specific manner (Llave *et al.*, 2002; Brodersen *et al.*, 2008).

MIR genes are transcribed by RNA polymerase II (pol II) to produce a pri-miRNA with characteristic hairpin structures (Ambros *et al.*, 2003; Xie *et al.*, 2005a) (reviewed in Rogers and Chen, 2013). As protein-coding genes, the transcription of these *MIR* genes is dependent on the presence of distinct *cis* elements in the promoter region of the *MIR* gene, which are recognized by specific transcription factors (Megraw *et al.*, 2006; Zhao *et al*., 2013). The RNAse III DICER-like proteins (DCL), typically DCL1, is responsible of the processing of pri-miRNAs into smaller stem-loop structures, or precursor-miRNAs (pre-miRNA), which are further processed into miRNA duplexes (miRNA-5p/miRNA-3p) (**Figure 7**). The stem-loop structure is essential to guide the DCL-mediated cleavage of the pre-miRNA (Bologna *et al.*, 2009; Song *et al*., 2010; Werner *et al.*, 2010). In the nucleus, miRNAS are methylated in the 3' region by HUA ENHANCER 1 (HEN1) preventing miRNA degradation (Yu *et al.*, 2005; Molnár *et al.*, 2007; Abe *et al.*, 2010). The protein HASTY (HST) is responsible of the translocation of miRNAs from the nucleus to the cytoplasm (Mee *et al.*, 2005). Once in the cytoplasm, the mature single-stranded miRNA is loaded into the RNA-induced silencing complex (RISC) containing an ARGONAUT (AGO) protein which have slicer activity (Mi *et al.*, 2008; Takeda *et al.*, 2008).

Once in RISC, the miRNA recognizes target transcripts by sequence complementarity (**Figure 7**).

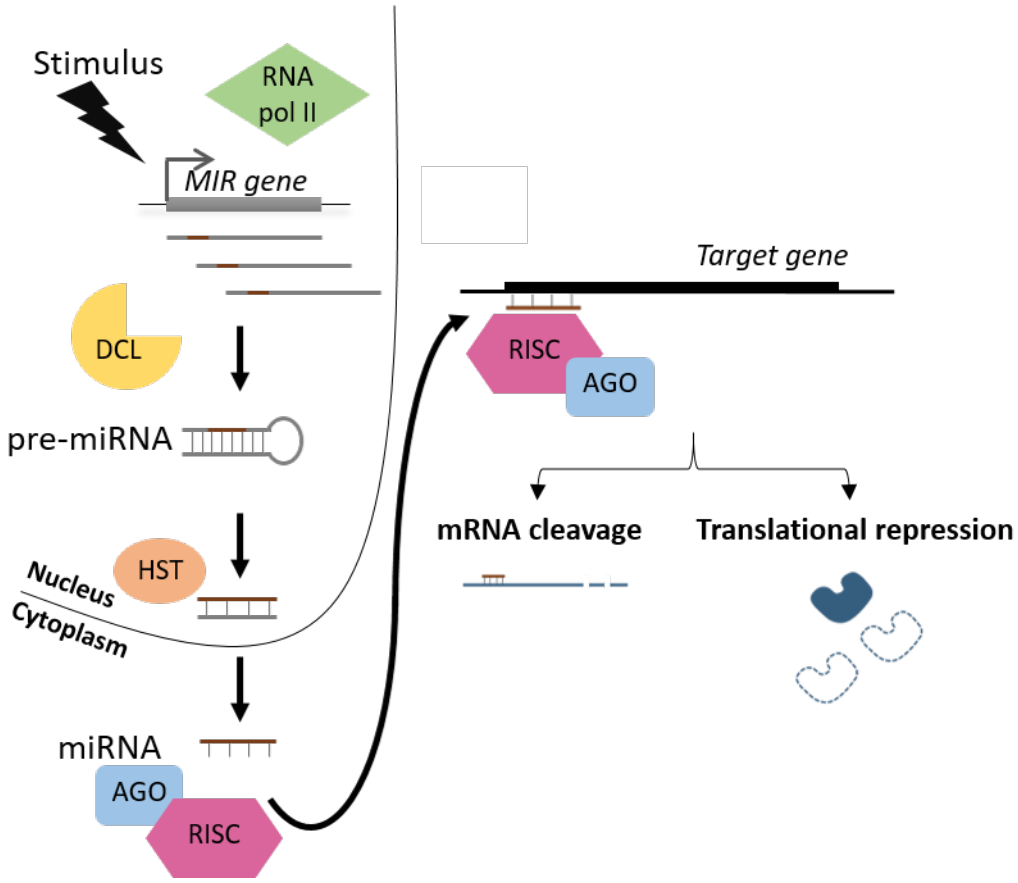

Figure 7: miRNA biogenesis and function in plants. *MIR* genes are transcribed by RNA polymerase II as long precursor transcripts that are sequentially processed by DCL proteins to generate miRNA duplexes. The miRNA duplex is translocated from the nucleus to the cytoplasm where the functional strand of the duplex is loaded into the RISC complex (RNA-Induced Silencing Complex). In RISC, the Argonaute protein (AGO) possesses mRNA slicing activity and miRNAs guide the cleavage or translational repression of target genes by sequence complementarity.

Contrary to miRNAs, which are produced from imperfect base-paired hairpin structures, siRNAs are produced from long, perfectly paired double-stranded RNA precursors. These sRNAs play a role in growth and developmental processes, as well as in the regulation of plant response to abiotic and biotic stresses (Borsani *et al.*, 2005; Katiyar-Agarwal *et al.*, 2006).

The introduction of next generation sequencing techniques for the analysis of small RNA populations has allowed the identification of a high number of miRNAs in plants. The miRBase is an repository of miRNA sequences and annotations

(http://www.mirbase.org; Kozomara *et al*., 2019). Based on their nucleotide sequence, miRNAs have been categorized into different families, each family comprising one or more members. Currently, the miRBase contains 738 mature rice (*O. sativa*) miRNAs, only after *Medicago truncatula* and *Glycine max* (756 miRNAs in both species). *A. thaliana* has 428 mature miRNAs annotated (miRBase release 22.1). Although a large number of miRNAs are identified in different plant species, the function of most of these miRNAs remains unknown.

6.2. Function of plant microRNAs

Plant miRNAs play a critical role in controlling diverse developmental processes and adaptation to environmental stress, both biotic and abiotic stresses (Jeong and Green, 2013; Seo *et al.*, 2013; Staiger *et al.*, 2013; Kumar, 2014; Weiberg *et al.*, 2014; Li and Zhang, 2016). In rice, miRNAs have been shown to control traits of agronomic importance, such as tillering, flowering, panicle an grain production (Zhang *et al.*, 2013; Hu *et al.*, 2018). Some miRNAs are known to be involved in developmental processes in Arabidopsis, such as miR393 (targeting the auxin receptor *TIR1* involved in auxin signaling) and miR171 (targeting APETALA2 controlling floral organ identity), among others (Chen *et al.*, 2011; Ma *et al.*, 2014). miR393 also plays a role in Arabidopsis immunity (Navarro *et al.*, 2006).

There are also miRNAs that are regulated by abiotic stresses, such as drought, cold, salinity, or UV radiation (Ruiz-Ferrer and Voinnet, 2009; Khraiwesh *et al*., 2012; Jeong and Green, 2013). For instance, the Arabidopsis miR397 or miR393 are induced by drought conditions (Sunkar and Zhu, 2004), the last one also in rice (Zhao *et al.*, 2007). miR393 has been associated to salt stress (Xia *et al.*, 2012) and miR169, miR319 or miR396 in cold stress (Sunkar and Zhu, 2004; Wang *et al.*, 2008; Zeng *et al.*, 2018). During stressful situations, plants increase the production of ROS, process that is also regulated by miRNAs, such as miR398 (Dugas and Bartel, 2008; Li *et al.*, 2011), or miR528 in rice (Wu *et al.*, 2017). miR168 and miR162 (targeting AGO1 and DCL1, respectively) control small RNA biogenesis and functioning (Xie *et al.,* 2003). miRNAs involved in biotic stress are discussed below (Section 6.2).

Finally, the role of miRNAs in nutrient homeostasis is well documented (Liang *et al*., 2015; Paul *et al*., 2015). Some of them are specifically responsive to a particular nutrient, but others can be regulated by different nutrients. For instance, 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). As previously mentioned, miR399 and miR827 are key players in the plant response to Pi starvation. Here, it is worth mentioning that most research on miRNAs involved in nutrient homeostasis focused on plants under nutrient-limiting conditions, and less is known about the implication of miRNAs in plants under nutrient excess. 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 unknown.

6.3. Regulatory role of microRNAs in plant immunity

The role of miRNAs in disease resistance was first described in the model plant *Arabidopsis thaliana* during interaction with the bacterial pathogen *P. syringae* (Navarro *et al.*, 2006). Here, perception of the *P. syringae* flagellin peptide flg2 induces miR393 accumulation, resulting in down regulation of auxin receptors and bacterial resistance. Since then, other miRNAs have been shown to play a key role in Arabidopsis immunity. Indeed, most of our current understanding on miRNAs in plant immunity comes from studies on the Arabidopsis/*P. syringe* pathosystem. Less is known about miRNAs involved in disease resistance in other plant species and/or during interaction with other types of pathogens.

Some examples of miRNAs playing a role in Arabidopsis immunity are: miR160, miR167, miR393, miR396, miR398b, miR400, miR472, miR773, miR844 and miR858, among others (Staiger *et al.*, 2013; Weiberg *et al.*, 2014; Baldrich *et al.*, 2015)(**Table 1**).

Studies in our group on miRNAs involved in Arabidopsis immunity revealed a function of miR396, miR773 and miR858 in the host response to infection by fungal pathogens (Camargo-Ramírez *et al*., 2017; Soto-Suárez *et al.*, 2017; Salvador-Guirao *et al*., 2018). miR396 targets transcription factors belonging to the Growth-Regulating Factor (GRF) family in many plant species. Arabidopsis lines in which miR396 activity is reduced using

artificial miRNA target mimics (*MIM* lines) confers resistance to necrotrophic (*P. cucumerina, Botrytis cinerea*) and hemibiotrophic fungal pathogens (*F. osysporum* f. sp. *conglutinans*, *C. higginsianum*), whereas *MIR396* overexpression results in increased susceptibility. miR396 functions as a negative regulator of defense responses (Soto-Suárez *et al.*, 2017). As for miR773 (targeting *MET2*, *METRHYLTRANSFERASE2*), this miRNA has been shown to function as a negative regulator of Arabidopsis immunity against the fungal pathogens *P. cucumerina*, *F. oxysporum* f. sp. *conglutinans* and *C. higginsianum* (Salvador-Guirao *et al.*, 2018). Finally, miR858 negatively regulates PTI against fungal pathogens by modulating the expression of Flavonoid-specific MYB transcription factor genes (Camargo-Ramírez *et al*., 2017).

A few number of miRNAs has been functionally characterized in the rice/*M. oryzae* interaction (**Table 1**). Some of these miRNAs function as positive regulators of immune responses (miR7695, miR160a, miR162a, miR398b, miR166k-166h and miR812w), whereas other miRNAs function as negative regulators of rice immune responses (miR156fhl, miR164a, miR167d, miR169a, miR319b, miR396, miR399, miR444b.2, miR439 and miR1873) (Campo *et al.*, 2013; Li *et al.*, 2017; Salvador-Guirao *et al.*, 2018b; Wang *et al.*, 2018; Zhang *et al.*, 2018; Chandran *et al.*, 2019; Li *et al.*, 2019; Sánchez-Sanuy *et al.*, 2019; Bundó *et al.*, 2020; Campos-Soriano *et al.*, 2020; Zhou *et al.*, 2020; Campo *et al.*, 2021; Feng *et al.*, 2021; Junhua *et al.*, 2021) (**Table 1**). Regarding miR398, this particular miRNA functions in the plant response to pathogen infection as well as in abiotic stress responses by regulating the expression of genes involved in protection against oxidative stress (e.g. the copper superoxide dismutases CSD1 and CSD2, and Cu chaperones for SOD1) (Sunkar *et al*., 2006; Beauclair *et al*., 2010). To note, miR398 has been reported to function as a positive regulator of immune responses in rice against *M. oryzae* while negatively regulating immune responses against *P. syringae* in Arabidopsis (Li *et al.*, 2014).

In other studies, the activation of *MIR166k-166h* encoding a polycistronic miRNA from rice was found to enhance resistance to infection by *M. oryzae* and *F. fujikuroi* in rice (Salvador-Guirao *et al*., 2018). The regulatory role of miR166k-166h relies in the activity of miR166k-5p (encoded by the miR166k-166h precursor) on *EIN2* (*ETHYLENE INSENSITIVE 2*) transcripts, the *EIN2* gene being a central transducer in the ethylene

signaling pathway in rice plants. (Helliwell *et al*., 2016). Regarding miR812, a new member of the miR812 family in rice, miR812w, its involvement in blast resistance was recently described by our group (Campo *et al.*, 2021). In addition to miRNAs themselves, components of the miRNA biogenesis machinery also play a role in blast resistance. Whereas silencing of *OsDCL1* enhances resistance to the rice blast fungus, its activation compromises resistance against this fungus (Zhang *et al.*, 2015; Salvador-Guirao *et al.*, 2019). Clearly, the identification and functional characterization of miRNAs playing a role in disease resistance in rice opens the possibility of using miRNAs as biomarkers in the management of disease resistance in rice breeding programs.

Plant miRNAs play a role not only in PTI but also in ETI responses. As for ETI, there is a set of miRNAs, especially those 22 nt in length, that target resistance (R) genes (e.g. intracellular nucleotide-binding/leucine rich-repeat receptors, or NLRs) and trigger the production of phasiRNAs from their target mRNAs (Song *et al.*, 2021). These phasiRNAs further target NLR genes for cleavage to suppress NLR gene function (Fei *et al*., 2013). These miRNAs are highly conserved in different species, and most of them belong to the miR482/2118 superfamily (Canto-Pastor *et al.*, 2019). This miRNA-NLR-siRNA regulatory mode is an effective way for plants to prevent the autoimmunity and growth inhibition caused by unregulated R gene expression in the absence of pathogens (Deng *et al.*, 2018). phasiRNAs derived from conserved regions of the NLR genes increase the number of genes targeted by a single miRNA, thus enhancing its silencing effect on NLR genes (Fei *et al*., 2013).

The current picture is that plant miRNAs can function as positive or negative regulators of immune responses, depending on the target gene that they regulate (Zhang *et al.*, 2019). Even though an important number of miRNAs has been shown to be regulated by pathogen infection in plants, the biological role of these pathogen-regulated miRNAs in plant immunity is not completely understood.

miRNA		Target gene	Regulation
A. thaliana	miR393	TIR1/AFB2/AFB3	Positive
	miR160	ARFs	Positive
	miR167	ARFs	Positive
	miR396	GRFs	Negative
	miR398b	CSD	Negative
	miR400	PPR1/PPR2	Negative
	miR472	RPS5	Negative
	miR773	MET2	Negative
	miR844	CDS3	Negative
	miR858	MYB11/MYB12/MYB111	Negative
O. Sativa	miR7695	*NRAMP6*	Positive
	miR160a	*ARFs*	Positive
	miR162a	*DCL1*	Positive
	miR398b	*CSD2/CSD1*	Positive
	miR166k-166h	*EIN2*	Positive
	miR812w	*LRR/ACO3/CIPK10*	Positive
	miR156fhl	*SPLs*	Negative
	miR164a	*NAC60*	Negative
	miR167d	*ARF12*	Negative
	miR169a	*NF-YAS*	Negative
	miR319b	*TCP21*	Negative
	miR396	*GRFs*	Negative
	miR399	*PHO2*	Negative
	miR444b.2	*MADs*	Negative
	miR439	*LOC_Os01g23940* *LOC_Os01g36270*	Negative
	miR1873	*LOC_Os05g01790*	Negative

Table 1: miRNAs with a known function in Arabidopsis and/or rice immunity. The miRNA/target gene and the function of the corresponding miRNA (positive or negative regulator of immune responses) are indicated. ACO, 1-aminocyclopropane-1-carboxylase oxidase; ARF, Auxin Response Factor; AFB, Auxin Signaling F-Box2; CDS, Cythidinaphpsphate Diacylglycerol Synthase3; CIPK, Calcineurin B Like (CBL)-interacting serine– threonine protein kinase; CSD, Cu/Zn-Superoxide dismutase; DCL1, Dicer-like Protein; EIN, Ethylene-insensitive Protein; GRF, Growth regulating factors; LRR, Leucine-rich repeat; MET, Methyltransferase; MYB, V-myb

myeloblastosis viral oncogene homolog; NAC, No apical meristem; NF-YA, Nuclear transcription factor Y subunit alpha; NRAMP, Natural resistance-associated macrophage protein; PHO2, Phosphate2; PPR, pentatricopeptide repeat; RPS5, Resistant to Pseudomonas syringe; SPL, SQUAMOSA promoter-binding protein-like; TCP, Teosinte Branched1, Cycloidea, and Proliferating Cell Nuclear Antigen binding factor; TIR, Transport inhibitor response 1-like protein.

7. Impact of nutrient stress on plant immunity

Nutrient stress caused by excess or deficiency of nutrients might affect the plant's response to pathogen infection, thus, determining the outcome of the interaction, resistance or susceptibility. Moreover, diverse results are found in the literature on the effect of nutrient stress on disease severity, with both increased and decreased disease severity depending on the identity of the interacting partners (host and pathogen) (Veresoglou *et al.*, 2013). Although adaptation to nutrient stress and immunity are not independent processes, the molecular mechanisms involved in nutrient responses and innate immunity have been so far investigated separately from each other. To better understand how plants defend themselves from pathogen attack, it is essential to consider that they can be exposed to adverse environmental conditions, including improper nutrient supply and nutritional imbalances. In the case of foliar pathogens, the pathogen entirely depends on the host for supply of nutrients and the availability of host nutrients appears to be an important factor contributing to the establishment of the disease, as it is the case of the rice blast disease (Wilson *et al.*, 2012).

Plants can develop different nutritional-based defense mechanisms based on sequestration of essential nutrients away from the invading pathogen (withholding strategies), or localized accumulation of nutrients at the infection site that become toxic to the pathogen (i.e. hyper-accumulation of Fe for activation of an oxidative burst toxic for the pathogen). The term "nutritional immunity" is used in human health as a non-specific host immune response and refers to mechanisms for withholding iron availability by the host that becomes inaccessible to the pathogen (Hood and Skaar, 2012). The term nutritional immunity can be also applied to restriction mechanisms of essential nutrients in plants to defend against pathogens. In plant/pathogen interactions, there is a competition between the host and the pathogen for essential nutritional resources, as the pathogen must acquire nutrient elements from host tissue,

and reciprocally, the host plant might defend itself by depriving the invader of nutritional resources. This dynamic interaction between pathogens and host 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. However, the massive use of fertilizers might have a negative impact on nutrient use efficiency in the host plant and might be responsible of metabolic changes in the host that jeopardize the plant defense responses. On the contrary, the accumulation of certain nutrients that can be toxic for the pathogen (e.g. iron) might protect the plant from infection.

It is generally assumed that N nutrition has an effect on disease incidence in different plant species (Sun *et al.*, 2020). During infection, pathogens require N sources, including NH_4^+ and NO_3^-, as well as amino acids. It has long been recognized that high N fertilization enhances susceptibility to the blast fungus in rice plants (Ballini *et al*., 2013). In Arabidopsis, N starvation conditions reduce plant susceptibility against the necrotrophic pathogen *Botrytis cinerea* (Fagard *et al.*, 2014). 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 primary and secondary metabolism, which, in turn, might affect host defense responses. While promoting plant growth, high N input might result in less formation of lignin and reduction in the thickness of the secondary cell wall, the plant's physical barrier to pathogen infection (Zhang *et al.*, 2017). From the perspective of the pathogen, fungal pathogenicity can be affected by N availability. The nitrogen-induced susceptibility (NIS) to rice blast has been shown to be associated with the induction of rice genes implicated in N recycling and an increase of *M. oryzae* pathogenicity (Huang *et al.*, 2017) (**Figure 8**). Contrary to what it is observed in rice, N fertilization 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 (Veresoglou *et al.*, 2013).

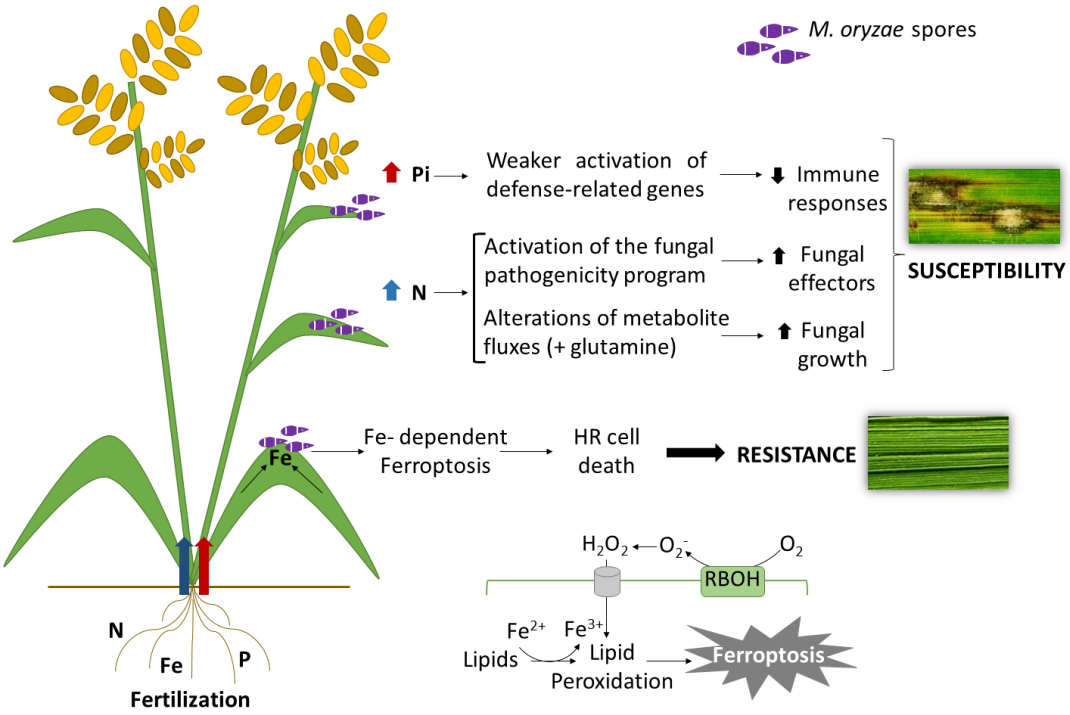

Figure 8. Crosstalk between nutrient signalling pathways and immune response. Nutrients are essential for plant growth and development. They are obtained from the soil through the plant root system. However, insufficient or *excessive* amounts of certain nutrients can affect disease resistance in plants. In rice, high nitrogen (N) supply not only promotes *M. oryzae* growth but also induces the expression of fungal effector genes, thus making the plant more susceptible to infection [(Ballini, Nguyen and Morel, 2013)]. Growing rice plants under high phosphate (Pi) conditions leads to an increase in leaf Pi content, which is associated with a weaker activation of defence responses and susceptibility against the rice blast fungus [(Campos-Soriano *et al.*, 2020)]. Ferroptosis, an iron (Fe)- and ROS-dependent cell death process, contributes to arrest *M. oryzae* infection in incompatible rice/*M. oryzae* interactions [(Dangol *et al.*, 2019)]. Thus, iron levels must be strictly controlled in the host plant to *maintain* the subtle balance between *growth* and *defence.*

Regarding Pi nutrition, emerging evidence supports the existence of crosstalk between the Pi starvation signaling and immune signaling in plants (Castrillo *et al.*, 2017; Chan *et al.*, 2021). Genome-wide gene expression analysis revealed that *PHR1*, master regulator of PSR in Arabidopsis plants, negatively regulates immune responses (Castrillo *et al.*, 2017). The *phr1* mutant lines showed enhanced resistance to infection by the bacterial pathogen *P. syringae* DC3000 and the oomycete pathogen *Hyaloperonospora arabidopsidis* (Castrillo *et al.*, 2017). Transgenic expression of a phytoplasma effector (SAP11) in Arabidopsis induced PHR1-dependent Pi starvation responses, thus causing the plants to be more susceptible to *P.syringae* pv.tomato DC3000 infection (Lu *et al.*, 2014). The PSR system also seems to control root colonization by the endophytic fungus

Colletotrichum tofieldiae in Arabidopsis (Hiruma *et al.*, 2016). On the other hand, our knowledge of interactions between plant adaptive mechanisms to Pi excess and immunity is still limited. Only recently, it was described that high Pi fertilization compromises the expression of immune responses and enhances susceptibility to infection by *M. oryzae* in rice plants (Campos-Soriano *et al.*, 2020) (**Figure 8**). In other studies, overexpression of the *OsPT8* phosphate transporter, and subsequent increase in Pi content, was reported to enhance susceptibility to *M. oryzae* infection (Dong *et al.*, 2019). Therefore, over-use of fertilizers in rice fields might have unintended consequences in rice production by facilitating blast infection, while contributing to water and soil pollution.

Root colonization by arbuscular mycorrhizal (AM) fungi helps the plant to enhance nutrient uptake, mainly P and N (Parniske, 2008; Bonfante and Genre, 2010; Maclean *et al*., 2017; Choi *et al*., 2018). Improvement in plant nutrition by AM fungi has beneficial consequences to the host plant in terms of growth and productivity. AM colonization also improves resistance to biotic and abiotic stress in several plant species (Campo *et al.*, 2020). It has been demonstrated that inoculation of rice plants with the AM fungus *Rhizophagus irregularis* confers protection to the rice blast fungus, the level of mycorrhiza-induced blast resistance varying among rice varieties (Campo *et al.*, 2020).

As previously mentioned, Fe is an essential element for plants and a co-factor for a variety of proteins mediating redox reactions. When in excess, however, iron becomes toxic to the plant cells due to the formation of ROS, which might cause oxidative damage to macromolecules and cellular structures, eventually leading to death. In plant-pathogen interactions, iron homeostasis must be carefully regulated to allow normal plant growth while providing a way to arrest pathogen growth. Evidence supports that iron content has an effect on the expression of immune responses in plants, including rice (Peris-Peris *et al.*, 2017; Verbon *et al.*, 2017; Sánchez-Sanuy *et al.*, 2019; Herlihy *et al*., 2020). In *M. oryzae*-infected rice leaves, Fe has been found to accumulate at the sites of attempted penetration by the fungus (appresoria), and in cells in the vicinity of the infection site (Sánchez-Sanuy *et al.*, 2019). Studies in wheat plants revealed that infection with *Blumeria graminis* f. sp. *tritici* elicits Fe accumulation in the apoplast which in turn mediates apoplastic oxidative burst and induction of defense gene expression

(**Figure 8**) (Liu *et al.*, 2007). It has been proposed that disturbance of Fe homeostasis is sufficient to prime the plant immune system for enhanced defense (Trapet *et al.*, 2020).

Cu is an important element in a number of pesticides in agriculture because it interferes with numerous enzymatic reactions and blocks respiratory activity in bacterial, fungal and oomycete pathogens. Accordingly, Cu is widely used in crop protection because of its antimicrobial activity against phytopathogens. In rice, Cu application is very effective to suppress bacterial blight, caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*). Studies in Arabidopsis grown under different Cu concentrations showed increased expression of salicylic- and ethylene-dependent genes and, therefore, increased resistance to *P. syringae* (Liu *et al.*, 2015). Because of the wide application of Cu-containing pesticides in agriculture, Cu-resistant pathogenic bacteria have been reported (Yuan *et al.*, 2010).

In summary, evidence gathered over years indicates that nutrient stress might impact disease resistance. Our understanding of the molecular mechanisms underlying the innate ability of plants to cope with pathogen infection in heterogeneous environments is, however, far from complete. Further investigation on these processes in different plant/pathogen/environment interactions would provide a solid foundation for the development of novel strategies for rationally optimizing fertilizer and pesticide use in crops production and protection against diseases.

8. miRNAs in the cross-talk between nutrient stress and immune signaling

Understanding how plants cope with pathogens under nutrient stress conditions and how nutrient signaling pathways interact with immune signaling is an issue of great importance in crop protection. Clearly, a dynamic regulation of gene expression is required for optimal plant responses to nutrient conditions that are diverse in time and space, as well as during pathogen infection. Being versatile regulators of gene expression, miRNAs are well suited for proper integration of these inputs.

In rice, miR7695 has been reported to regulate iron homeostasis and rice immunity. miR7695 negatively regulates the expression of *OsNramp6* (*Natural resistance-*

associated macrophage protein 6), encoding an iron and manganese transporter from rice (Campo *et al.*, 2013; Peris-Peris *et al.*, 2017). miR7695 regulates not only Fe content, but also defense responses during infection of rice plants with *M. oryzae*. Thus, *MIR7695* activation is accompanied by a superinduction of *PR* and diterpenoid phytoalexin biosynthesis genes which, in turn, results in enhanced resistance to infection by *M. oryzae* (Sánchez-Sanuy *et al.*, 2019).

Increasing evidence supports that *MiR399* functions as an integrator of Pi starvation and immune responses in Arabidopsis (Castrillo *et al.*, 2017; Chan, 2021). Results obtained in our laboratory indicated that miR399 functions as a regulator of immune responses in rice plants. Here, *MIR399* overexpression in rice increases Pi content in leaves (Campos-Soriano *et al.*, 2020). A weaker induction of defense-related genes occurs in *MIR399* overexpressor plants which results in enhanced susceptibility to *M. oryzae* infection (Campos-Soriano *et al.*, 2020). A phenotype of blast susceptibility is also observed in rice plants that have been grown under high Pi supply (Campos-Soriano *et al.*, 2020).

Thus, from what we have learned so far, miRNAs can play a role in crosstalk between nutrient and immune signaling. miRNAs might potentially work as molecular switches between these two intimately connected processes. As miRNAs are capable of fine-tuning target gene expression, rather than on-off regulations, these riboregulators are well suited to function in the crosstalk between signaling pathways induced by different types of stress. At present, however, our knowledge on interconnected regulations between nutrient signaling and immune signaling in which miRNAs participate is still in its infancy. The use of miRNAs as biomarkers for nutrient stress and disease resistance remains an interesting research field with applications in breeding programs.

Nowadays, fertilizers and pesticides are used to improve crop yield and to protect crops from diseases caused by pathogens. However, the indiscriminate use of fertilizers might have negative effects on disease resistance. This is well illustrated by the fact that N or Pi over-fertilization enhances susceptibility to infection by the blast fungus in rice. Ironically, under a scenario of increased blast susceptibility due to N/P fertilization, more pesticides need to be applied in rice fields to reduce losses caused by pathogen

infection. In addition to its environmental and health impact, the massive application of fertilizers and pesticides might negatively affect nutrient use efficiency in modern cultivars. The development of appropriate nutrition and management practices represents the basis for sustainable crop production. Climate change also influences the ecology of pathogens and diseases, with possible implications for crop protection and pesticide use. A better knowledge on the molecular mechanisms involved in plant adaptation to nutrient stress and pathogen infection is crucial to define innovative strategies aiming the transition to sustainable crop production systems.

The rational for conducting this research came from data previously obtained in our group on small RNA sequencing of rice tissues treated with elicitors obtained from the rice blast fungus *M. oryzae*. In that study, a large set of pathogen-regulated miRNAs potentially involved in rice immunity was identified. Among them, there were several miRNAs with a known function in nutrient homeostasis, such as miR399 and miR827 (phosphate homeostasis), miR395 (sulfur homeostasis) or miR398 (Copper homeostasis) (Campo *et al.*, 2013; Baldrich *et al.*, 2015) .

Even though results obtained in our group and results from other groups provided a large amount of information supporting the existence of links between Pi signaling and defense signaling in Arabidopsis and rice, it is unknown how these signaling pathways interact during infection. This PhD Thesis aimed to increase our knowledge in this topic. In particular, I have investigated how Arabidopsis and rice plants cope with pathogens under Pi stress and how miR399 and miR827 (miRNAs typically associated to Pi signaling), and their corresponding target genes, modulate the expression of immune responses to infection by fungal pathogen.

Objectives

Objectives

The main objective of this PhD thesis was to study the regulatory role of miR399 and miR827, and their corresponding target genes, in the crosstalk between phosphate homeostasis and immune responses in Arabidopsis and rice.

The specific objectives were the following:

1. To determine the function of miR399 and its target gene *PHO2* (*PHOSPHATE 2*) in the control of Pi homeostasis and immune responses in Arabidopsis, and to examine how Pi content might affect Arabidopsis immune responses. Results obtained in this study are presented in **Chapter I**.

2. To determine the function of miR827 and its target gene *NLA* (*NITROGEN LIMITATION ADAPTATION*) in the control of Pi homeostasis and immune responses in Arabidopsis. Results obtained in this study are presented in **Chapter II**.

3. To study the contribution of Pi compartmentalization between the vacuoles and cytoplasm of rice plants in the context of disease resistance. Generation and characterization of loss-of-function rice mutants in the *MIR827* locus using the CRISPR-Cas9 system. Results are presented in **Chapter III**.

CHAPTER I

Phosphate-induced production of reactive oxygen species regulates immune responses and resistance to pathogen infection in Arabidopsis

Results presented in this chapter are included within a manuscript submitted for publication

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ABSTRACT

In nature, plants are concurrently exposed to a number of abiotic and biotic stresses. Our understanding of convergence points between responses to combined biotic/abiotic stress pathways remains, however, rudimentary. Here we show that *MIR399* overexpression, loss-of-function of *PHO2* (*PHOSPHATE2*), or treatment with high Pi, is accompanied by an increase in phosphate (Pi) content and accumulation of reactive oxygen species (ROS) in *Arabidopsis thaliana*. High Pi plants (e.g. miR399 overexpressor, *pho2* mutant, and plants grown under high Pi supply) exhibited resistance to infection by necrotrophic and hemibiotrophic fungal pathogens. In absence of pathogen infection, the high Pi plants showed a basal expression of genes involved in salicylic acid (SA) and jasmonic acid (JA) signaling pathways which is consistent with increased levels of SA and JA in plants that overaccumulate Pi. These plants also show induction of SA- and JA-regulated defense responses upon pathogen challenge. During infection, however, an opposite regulation in the two branches of the JA pathway (ERF1/PDF1.2 and MYC2/VSP2) occurs in high Pi plants. While the ERF1-PDF1 branch positively responds to fungal infection, the MYC2/VSP2 branch is negatively regulated during infection in high Pi plants. This study provides insights into how the Pi status of Arabidopsis plants influences hormone levels to promote resistance to fungal pathogens, while providing a basis to better understand how Pi and pathogen-induced signaling pathways are integrated for modulation of plant immune responses. These findings also support that miR399 plays a positive role in Arabidopsis immunity via regulation of Pi content.

INTRODUCTION

In nature, plants are simultaneously exposed to a combination of biotic and abiotic stresses that are diverse in time and space, which requires proper integration and crosstalk between different stress response pathways. Exposure to a single stress might, however, impact the plant response to another stress (Kissoudis *et al.*, 2014; Nejat and Mantri, 2017; Pandey *et al.*, 2017). For instance, plant immune responses are altered in plants exposed to drought or high salinity (Yasuda *et al.*, 2008; Atkinson and Urwin, 2012; Bostock *et al.*, 2014) while inappropriate supply of mineral nutrients (e.g. nitrogen supply) might also impact disease severity (Snoeijers *et al.*, 2000; Ballini *et al.*, 2013). However, the effect of combined abiotic and biotic stress factors might vary depending on the nature of these interactions, and the plant response to simultaneously or sequentially applied stresses can not be simply inferred from responses to individual stresses (Prasch and Sonnewald, 2013; Coolen *et al.*, 2016; Pandey *et al.*, 2017; Nobori and Tsuda, 2019). As stress responses are costly, when facing with multiple stresses simultaneously, plants need to prioritize their stress responses for efficient use of finite resources, in accordance with the optimal defense theory (ODT) (Meldau *et al.*, 2012). According to ODT, stress responses are prioritized in the most valuable parts (Keith and Mitchell-Olds, 2017), and recent findings indicate that Arabidopsis plants spatially separate contrasting stress responses in leaves of different ages (e.g. young leaves exhibit higher biotic stress responses but lower abiotic stress responses compared with old leaves) (Berens *et al.*, 2019; Wolinska and Berens, 2019). To date, little information is available on the molecular mechanisms by which biotic and abiotic stress responses are differentially prioritized in plants, and how they adapt to conflicting stresses for optimal responses.

To defend themselves against pathogens, plants have evolved an innate immune system in which many interconnected processes are involved (Jones and Dangl, 2006). Pathogen-induced pathways are defined principally according to the molecules recognized by the host plant (Jones and Dangl, 2006; Boller and Felix, 2009; Thomma *et al.*, 2011; Couto and Zipfel, 2016; Upson *et al.*, 2018). Plants recognize pathogen-associated molecular patterns (PAMPs) by receptors at the plasma membrane, which triggers the induction of multiple cascades leading to the induction of immune

responses, referred to as PAMP-triggered immunity (PTI). Components of PTI include the reinforcement of cell walls, accumulation of reactive oxygen species (ROS), activation of phosphorylation cascades, production of antimicrobial compounds and accumulation of pathogenesis-related (PR) proteins, among others (Andersen *et al.*, 2018). Some successful pathogens can overcome PTI by delivering effectors into plant cells that suppress PTI, thus, leading to disease susceptibility. In turn, some plants have evolved another immune response in which microbial effectors (or host proteins modified by effectors) are recognized by intracellular receptor proteins encoded by resistance (*R*) genes (Han, 2019). This recognition triggers a rapid and robust defense response, the so called effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI is often accompanied by a hypersensitive response (HR) at the infection site, a form of programmed cell death (Thakur *et al.*, 2019). However, some PAMPs (e.g. the bacterial Harping HrpZ protein, can also induce HR in plants (Chang and Nick, 2012).

Among ROS, H_2O_2 is relatively stable and is an important molecule 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. ROS also function as molecules for the activation of defense mechanisms, and triggers localized cell death around the infection site (Torres *et al.*, 2002). Phytohormones, together with ROS, provide important signals to help orchestrate plant responses to abiotic and biotic stresses. Immune responses are coordinated largely by the phytohormones salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) (Aerts *et al.*, 2021). Plant hormones do not function independently, but synergistic or antagonistic interactions between hormone pathways ultimately drive the fine-tuning of plant defense responses. At the organismal level, hormone crosstalk might also balance trade-offs between conflicting biotic and abiotic stress responses (i.e. prioritization of responses in leaves of Arabidopsis plants) (Berens *et al.*, 2019; Wolinska and Berens, 2019). Sugar and ROS have also been proposed as candidates signaling molecules to regulate prioritization between biotic and abiotic stress responses (Wolinska and Berens, 2019).

Plant miRNAs are a class of small RNA molecules that mediate post-transcriptional gene silencing through sequence complementarity with cognate target transcripts (Bartel,

2004; Xie *et al.*, 2005). They are transcribed from *MIR* genes as long precursor transcripts that adopt a stem-loop structure by self-complementarity that are processed by a RNase III DICER-like, typically DCL1, to produce a double stranded duplex, the miRNA-5p/miRNA-3p duplex (previously named as miRNA/miRNA*) (Borges and Martienssen, 2015). The functional strand of the duplex is selectively loaded into an ARGONAUTE (AGO) protein, the effector protein of the RISC (RNA-induced silencing complex), and guide post-transcriptional gene silencing via cleaving the target mRNA or by translational inhibition (Llave *et al.*, 2002; Brodersen *et al.*, 2008). The important role of plant miRNAs in diverse developmental processes and adaptation to environmental stresses is well documented (Chen, 2009; Seo *et al.*, 2013; Staiger *et al.*, 2013; Song *et al.*, 2019).

The first plant miRNA demonstrated to be involved in immunity was the Arabidopsis miR393. 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 (Jagadeeswaran *et al.*, 2009; Shivaprasad *et al.*, 2012; Seo *et al.*, 2013; Staiger *et al.*, 2013; Huang *et al.*, 2016). Some examples are: miR160, miR396, miR398, miR400, miR472, miR773, miR844, miR858, miR863, miR156 (Li *et al.*, 2010; Boccara *et al.*, 2014; Park *et al.*, 2014; Lee *et al.*, 2015; Niu *et al.*, 2016; Camargo-Ramírez *et al.*, 2017; Salvador-Guirao *et al.*, 2018; Yin *et al.*, 2019). Depending on their target gene, these miRNAs can function as positive or negative regulators in fine-tuning immune responses. Even though pathogen infection has been shown to induce alterations in the expression of a plethora of Arabidopsis miRNAs, the mechanistic role of these miRNAs in processes underling immunity is often unclear. Additionally, most studies on miRNAs involved in plant immunity have been carried out in the interaction of Arabidopsis plants with the bacterial pathogen *Pseudomonas syringae*, and less is known on miRNAs involved in resistance against fungal pathogens.

On the other hand, distinct miRNAs have been associated with regulation of nutrient homeostasis in plants (Paul *et al.*, 2015). Perhaps the best known example is miR399 involved in the control of phosphate (Pi) homeostasis in Arabidopsis plants (Chiou *et al.*,

2006). Under limiting Pi conditions, miR399 accumulation increases and causes repression of its target gene, *PHO2* (*PHOSPHATE2*) encoding an E2 ubiquitin-conjugating enzyme responsible of degradation of phosphate transporters (Fujii *et al.*, 2005; Chiou *et al.*, 2006; Liu *et al.*, 2012; Huang *et al.*, 2013; Kraft *et al.*, 2016). Hence, miR399 accumulation in response to Pi starvation relieves negative post-transcriptional control of Pi transporters and promotes uptake of Pi in Arabidopsis plants. The miR399/PHO2 regulatory module has been also recognized as a regulator of Pi homeostasis in rice (Chien *et al.*, 2017; Puga *et al.*, 2017).

In this work, we investigated whether miR399 plays a role in disease resistance in Arabidopsis. We show that miR399 overexpression and loss-of-function of *pho2* causes an increase in Pi level, these plants also exhibiting resistance to infection by both necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Colletotrichum higginsianum*) fungal pathogens. Growing Arabidopsis plants under high Pi supply also increases resistance to infection by these pathogens. In the absence of pathogen infection, plants that overaccumulate Pi (e.g. miR399 overexpressor, *pho2* mutant, wild-type plants grown under high Pi supply) show accumulation of ROS, higher SA and JA content, and up-regulation of genes involved in SA- and JA-dependent defense pathways. Pathogen infection is associated with higher production of ROS and stronger induction of SA- and JA-pathways in high Pi plants, which correlates well with increased levels of SA and JA, and resistance to pathogen infection. To note, superinduction of the ERF1 branch of the JA pathway, but repression of the MYC2/VSP2 branch, occurs in high Pi plants during pathogen infection. Overall, our results support that an increase in Pi content has an impact on SA and JA homeostasis and promotes resistance to infection by necrotrophic and hemibiotrophic fungal pathogens in Arabidopsis. These results are markedly different from those recently reported on rice (Campos-Soriano *et al.*, 2020) where miR399 overexpression and high Pi supply were found to enhance susceptibility to infection by the rice blast fungus *Magnaporthe oryzae*. These findings illustrate the need of investigating the effects of nutrient supply on the expression of immune responses and disease resistance on a case-by-case basis.

EXPERIMENTAL PROCEDURES

Plant material and infection assays

Arabidopsis thaliana (ecotype Columbia-0) plants were grown in a mixture of soil:perlite:vermiculite (2:1:1) and modified Hoagland half strength medium, under neutral photoperiod (12h light / 12h dark), 60% of humidity and a temperature of 22ºC ± 2ºC. The fungus *Plectosphaerella cucumerina* was grown on PDA (Potato Dextrose Agar) plates with chloramphenicol (34 µg/ml). *Colletotrichum higginsianum* was grown on Oatmeal agar plates in darkness. Fungal spores were collected by adding sterile water to the surface of the mycelium, and adjusted to the desired final concentration using a Bürker counting chamber.

For infection experiments in soil-grown plants, three-week-old plants were spray-inoculated with a spore suspension of *P. cucumerina* (5 x 10^5 spores/ml), or mock-inoculated. *P. cucumerina*-inoculated and mock-inoculated plants were maintained under high humidity and plant survival was assessed at 7 dpi. For infection with *C. higginsianum*, the fungal spores were locally-inoculated (4 x 10^6 spores/ml; 10 µl/leaf and 5 leaves/plant). Lesion area of *C. higginsianum*-infected leaves was measured with software ImageJ (National Institute of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij/) at 7 dpi. Three independent experiments were performed with at least 12 plants per genotype in each experiment. For *in vitro* experiments, two-week old Arabidopsis plants were spray-inoculated with *P. cucumerina* (4 x 10^6 spores/ml) or locally-inoculated with *C. higginsianum* (5 x 10^5 spores/ml). Fungal biomass was quantified by real-time PCR using specific primers for the corresponding fungus and the Arabidopsis *UBIQUITIN21* (At5g25760) gene as the internal control (Soto-Suárez *et al.*, 2017). PCR primers are listed in Table S1. Statistically significant differences were determined by t-test.

For Pi treatment experiments, plants were grown *in vitro* on meshes placed on agar plates with modified Hoagland half strength medium containing 0.25 mM KH_2PO_4 for one week. Seedlings were then transferred to fresh agar-medium at the desired concentration of Pi (0.05, 0.1, 0.25, or 2 mM Pi). The plants were allowed to continue

growing for one more week under each Pi regime. The *in vitro*-grown plants were then inoculated with a spore suspension of *P. cucumerina* or *C. higginsianum* as above.

The *pho2* mutant, previously named *UBC24* (UBIQUITIN-CONJUGATING ENZYME 24 (At2g33770; Columbia background) was obtained from the Arabidopsis Biological Resource Center (ABRC, ref. CS8508). A point mutation in the sixth exon (from G_{2539} to A, relative to the translational start codon) causes an early termination at the beginning of the UBC domain, thus, resulting in the loss of the ubiquitin-conjugating activity of PHO2 in the *pho2* mutant (Aung *et al.*, 2006).

Plant Tissue staining

For trypan blue staining, leaves were fixed by vacuum infiltration for 1h in ethanol:formaldehyde:acetic acid (80:3.5:5 v/v), stained with lactophenol blue solution for one hour, and then washed with chloral hydrate for 15 minutes. Leaves were placed on glass slides with glycerol and observed using a Leica DM6 microscope under bright field.

Diaminobenzidine tetrahydrochloride (DAB) staining was used to examine H_2O_2 levels. For this, Arabidopsis plants were immersed in a DAB solution (1 mg / ml) for 30 minutes with vacuum treatment, maintained during 4 hours in darkness and agitation, washed with 95% ethanol for 30 minutes at 75ºC, and observed using a Leica DM6 microscope under bright-field illumination.

For H_2DCFDA staining, the Arabidopsis leaves were placed on a solution of H_2DCFDA (at a concentration of 10µM), vacuum infiltrated during 5 minutes, and then maintained in darkness for 10 minutes. Two washes with distillated water were performed. Photographs were taken on a Leica DM6 microscope to visualize green fluorescence.

Generation of transgenic Arabidopsis plants

For *MIR399f* overexpression, the miR399 precursor sequence was cloned under the control of the *Cauliflower mosaic virus (CMV) 35S* promoter in the PMDC32 plasmid (Chiou *et al.*, 2006). The plant expression vector was transferred to the *Agrobacterium tumefacines* strain GV301. Arabidopsis (Col-0) plants were transformed using the floral dip method. Homozygosis was obtained by antibiotic selection. Segregation analysis confirmed transgene inheritance in successive generations of transgenic lines.

Measurements of Pi content and chlorophyll content

The Pi content of Arabidopsis plants was determined as previously described (Versaw and Harrison, 2002). Chlorophylls were extracted and quantified spectrophotometrically as previously described (Lichtenthaler and Buschmann, 2001).

Gene expression analyses

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from DNAse-treated total RNA (1 µg) with reverse transcriptase and oligo-dT (High Capacity cDNA reverse transcription kit, Applied Biosystems). RT-qPCR was performed in optical 96-well plates using SYBR® green in a Light Cycler 480 (Roche). Primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The *β-tubulin2* gene (At5g05620) was used to normalize the transcript level in each sample. Primers used for RT-qPCR and stem-loop RT-qPCR are listed in **Supplemental table S1**. Accumulation of mature miR399f was determined by stem-loop reverse transcription quantitative PCR (Varkonyi-Gasic *et al.*, 2007). Two-way analysis of variance (ANOVA) followed by HSD (Honestly-Significant-Difference) Tukey's test was used to analyze RT-qPCR data.

Hormone determination

The rosettes of three-week-old WT (Col-0), miR399 OE and pho2 plants were analyzed by LC-MS for SA, SAG, JA and OPDA content as previously described in Sánchez-Bel *et al.*, 2018. Briefly, 30 mg of freeze dried material was extracted with MeOH:H2O (30:70) containing 0.01% of HCOOH containing a mixture of 10 ug. L-1 of the internal standards salicylic acid-d 5 (SA-d5) and dehydrojasmonic acid (Sigma-Aldrich). Following extraction, samples were centrifuged (15.000 rpm, 15 min) and filtered through regenerated cellulose filters. An aliquot of 20 ul was injected into a UPLC (Waters Aquity) interfaced with a Xevo TQ-S Mass Spectrometer (TQS, Waters). Hormones were quantified by contrasting with an external calibrarion curve of pure chemical standards of SA, SAG, JA and OPDA. Sample separation was performed with a LC Kinetex C18 analytical column of a 5 µm particle size, 2.1 100 mm (Phenomenex). Cinematographic and TQS conditions were performed as described in Sanchez-Bel *et al.* (2018). At least 6 biological replicates were analyzed per genotype and condition, each replicate consisting of leaves from at least 3 independent plants. The plant material was lyophilized prior analysis. Two-way analysis of variance (ANOVA) followed by HSD (Honestly-Significant-Difference) Tukey's test was used to analyze data.

RESULTS

Resistance to fungal pathogens in Arabidopsis plants overexpressing miR399.

In Arabidopsis, the miR399 family comprises 6 members, *MIR399a-f.* Of them, miR399f has identical mature sequences in Arabidopsis and rice. Previous studies indicated that transgenic Arabidopsis and rice plants overexpressing miR399f overaccumulate Pi in leaves (Chiou *et al.*, 2006; Campos-Soriano *et al.*, 2020). In this work, we investigated whether miR399f overexpression, and subsequent Pi accumulation, has an effect on resistance to pathogen infection in Arabidopsis. Towards this end, transgenic plants overexpressing *MIR399f* (hereinafter referred to as miR399 OE plants) were generated. Compared to wild-type plants, miR399 OE lines accumulated precursor and mature miR399 sequences, which was accompanied by a decrease in *PHO2* transcript level, and overaccumulation of Pi in rosette leaves (**Figure 1a**).

The miR399 OE lines were challenged with the fungus *P. cucumerina*, the causal agent of the sudden death and blight disease in many dicotyledonous species. The Arabidopsis/*P. cucumerina* pathosystem emerged as the model system for studies on disease resistance against necrotrophic fungi (Ton and Mauch-Mani, 2004; Sánchez-Vallet *et al.*, 2012). At the time of pathogen inoculation (3-week-old plants), no obvious phenotypic differences were observed between miR399 OE plants and wild-type plants) (**Figure S1a, b**). However, at a later developmental stage, the miR399 and *pho2* plants displayed symptoms of Pi excess (e.g. chlorosis on mature leaves of adult plants, most probably, because of Pi accumulation overtime (results not shown; similar results were previously reported by (Chiou *et al.*, 2006).

Upon pathogen challenge, wild-type plants were severely affected by *P. cucumerina*, while the miR399 OE plants consistently exhibited enhanced resistance (**Figure 1b**). While 75-85% of miR399 OE plants survived at 7 dpi, only 20% of the wild-type plants were able to overcome infection (**Figure 1c**, left panel). Quantitative PCR (qPCR) measurement of fungal DNA confirmed less fungal biomass in leaves of miR399 OE plants compared with wild-type plants infected with *P. cucumerina* (**Figure 1c**, right panel), which is consistent with the phenotype of resistance that is observed in miR399 OE plants.

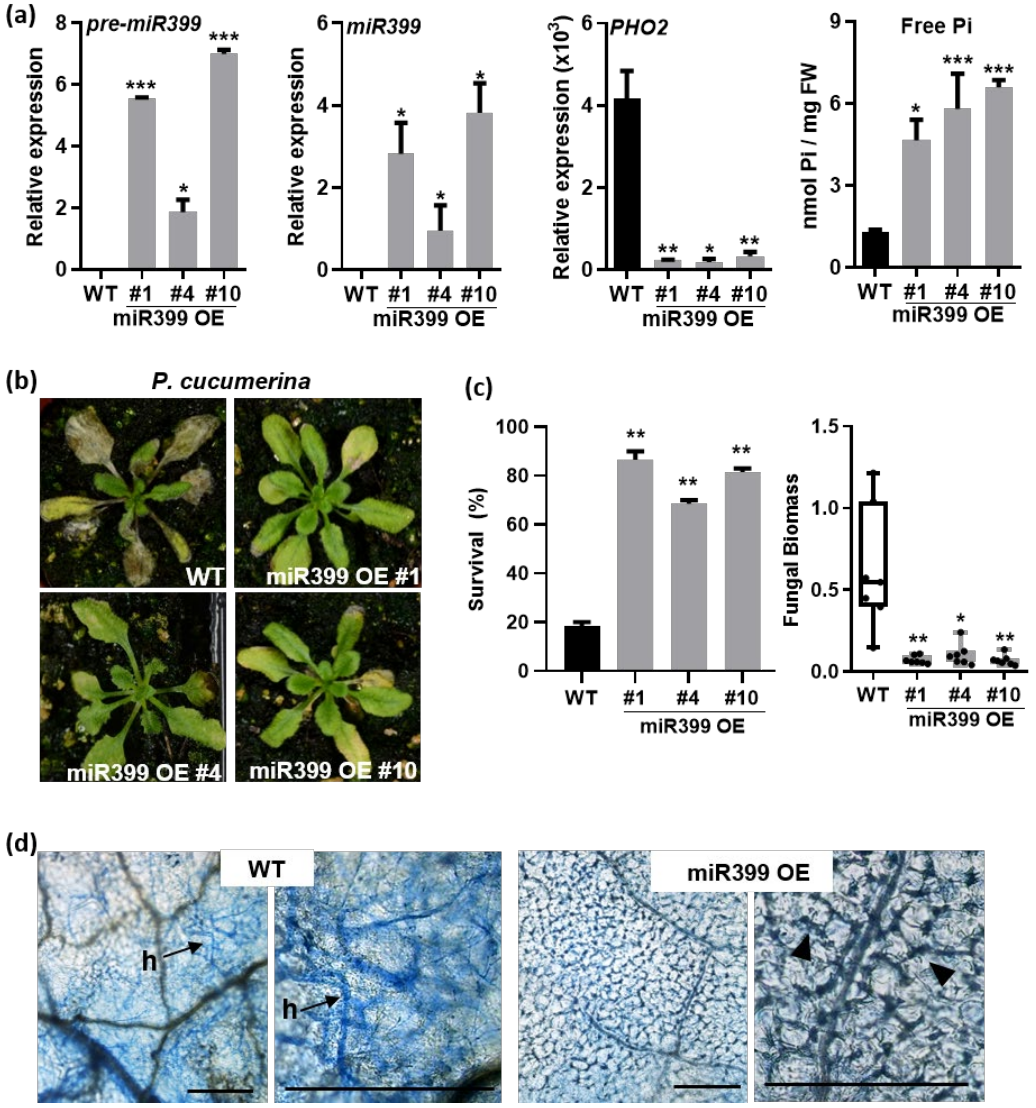

Figure 1. Resistance of miR399 OE plants to infection by the necrotrophic fungus *P. cucumerina*. MiR399 OE (homozygous lines #1, #4 and #10) and wild-type (WT) plants were grown in soil for three weeks. Three independent experiments were carried out with at least 12 plants/line each experiment. **(a)** Accumulation of precursor (pre-miR399) and mature miR399 sequences were determined by RT-qPCR and stem-loop RT-qPCR, respectively. Expression of the miR399 target *AtPHO2* by RT-qPCR. The accumulation of free Pi in leaves is shown (right panel). Bars represent mean ± SEM of 3 biological replicates with at least three plants per replicate (*t* test, $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$). **(b)** Plants were spray-inoculated with *P. cucumerina* spores (5 x 10^5spores/ml). Pictures were taken at 7 days post inoculation (dpi). **(c)** Survival ratio of WT and miR399 OE plants at 7dpi. Quantification of *P. cucumerina* DNA was performed by qPCR using specific primers of *P. cucumerina ß-tubulin* at 7 dpi. Values of fungal DNA were normalized against the Arabidopsis *UBIQUITIN21* gene (At5g25760). Comparisons have been made relative to WT plants. Data are mean ± SEM (n = 7) (*t* test, $*p \leq 0.05$, $**p \leq 0.01$). **(d)** Trypan blue staining of *P. cucumerina*-infected leaves of wild-type and miR399 OE plants (7 dpi). h, hyphae. Arrows and arrowheads indicate fungal hyphae and death cells, respectively. Higher magnifications are shown (WT and miR399 OE, right panels). Bars represent 300 µm.

Trypan blue staining was used to visualize both fungal structures and dead cells in the fungal-infected leaves of wild-type and miR399 OE plants. Whereas extensive fungal growth occurred in leaves of wild-type plants, no fungal growth could be observed in leaves of miR399 OE plants (**Figure 1d**, left panels). Instead, scattered groups of dead cells were visualized in *P. cucumerina*-infected miR399 OE plants (**Figure 1d**, right panels).

Since *P. cucumerina* is a necrotrophic fungus, we hypothesized that the effect of miR399 overexpression in disease resistance might be dependent on the life style of this pathogen. Accordingly, we investigated resistance of miR399 OE plants to infection by the hemibiotrophic fungus *Colletotrichum higginsianum*. This fungus causes the anthracnose leaf spot disease on *Brassica* species, including *A. thaliana* (O'Connell *et al.*, 2004). The miR399 OE plants showed resistance to *C. higginsianum* infection relative to wild-type plants, as revealed by quantification of diseased leaf area and fungal biomass (**Figure 2a**). As observed in infection assays with *P. cucumerina*, the *C. higginsianum*-infected miR399 OE plants exhibited groups of dead cells in their leaves (**Figure 2b**).

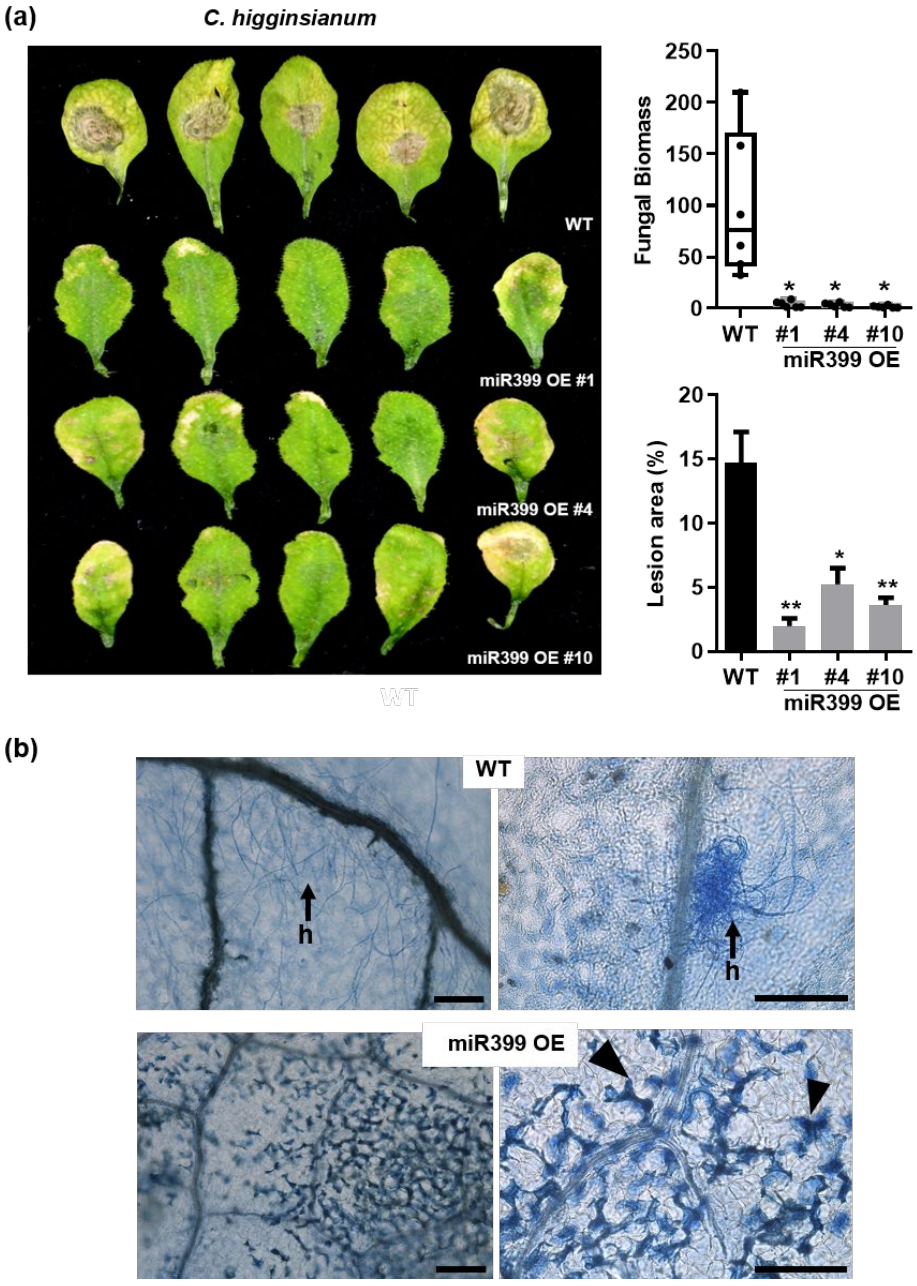

Figure 2. Resistance of miR399 OE plants to infection by the hemibiotrophic fungus *C. higginsianum*.

Leaves were locally inoculated with a spore suspension at 4 x 10^6 spores/ml. Results are from one out of three independent experiments performed with three independent miR399 OE lines (lines 1, 4 and 10) and wild-type plants which gave similar results. At least 12 plants/genotype were assayed in each experiment. **(a)** Disease symptoms at 7 days after inoculation with fungal spores. Diseased leaf area was quantified using image analysis software (ImageJ). Quantification of *C. higginsianum* DNA was carried out by qPCR using specific primers for the *C. higginsianum ITS2* (Internally transcribed spacer 2) gene at 7 days post inoculation (dpi). Values are fungal DNA levels normalized against the Arabidopsis *UBIQUITIN21* gene (At5g25760). Comparisons have been made relative to WT plants. Histograms show the mean ± SEM *(t* test, *$p \leq 0.05$; **$p \leq 0.01$). **(b)** Trypan blue staining of *C. higginsianum*-infected leaves of wild-type and miR399 OE plants at 7 dpi. h, hyphae. Arrows and arrowheads indicate fungal hyphae and cell death, respectively. Higher magnification of these regions are shown (right panels). Bars represent 100 µm.

Collectively, these results demonstrate that miR399 overexpression enhances resistance to infection by both necrotrophic (*P. cucumerina*) and hemibiotrophic (*C. higginsianum*) fungal pathogens. Hence, most probably, miR399-mediated resistance in Arabidopsis does not depend on the life style of the fungus. Interestingly, a pattern of cell death occurs in response to pathogen infection in miR399 OE plants.

pho2 mutant plants exhibit resistance to necrotrophic and hemibiotrophic fungi

miR399 targets and suppresses *PHO2* expression, this gene encoding the ubiquitin conjugating enzyme gene that mediates the degradation of Pi transporter proteins (Fujii *et al.*, 2005; Chiou *et al.*, 2006; Huang *et al.*, 2013). A loss-of-function allele of *PHO2* was previously described (Delhaize and Randall, 1995; Aung *et al.*, 2006). This mutant allele possesses a single nucleotide mutation that causes premature termination and loss of ubiquitin conjugation activity of PHO2 (**Figure S2a**). *pho2* resembles miR399 overexpressing plants in that they both show Pi overaccumulation in leaves resulting from increased Pi uptake from roots and root-to-shoot translocation (Aung *et al.*, 2006) (**Figure S2b**). We therefore hypothesized that loss-of-function of *PHO2* might result in similar disease phenotype as suppression of *PHO2* by overexpression of miR399.

The *pho2* plants were then examined for pathogen resistance. No phenotypic differences were observed between *pho2* and wild-type plants at the time of inoculation (**Figure S2c**). Consistent with the disease phenotype observed in miR399 OE plants, the *pho2* mutant exhibited resistance to infection by *P. cucumerina* (**Figure 3a**), which was confirmed by quantifying survival ratio of the infected plants and the amount of fungal biomass (**Figure 3b**). Trypan blue staining revealed a pattern of dead cells in leaves of *pho2* mutants that have been inoculated with *P. cucumerina* spores (**Figure 3c**), as it was also observed in miR399 OE plants. Finally, the *pho2* plants also showed resistance to *C. higginsianum* infection as compared with wild type plants (**Figure 3d**).

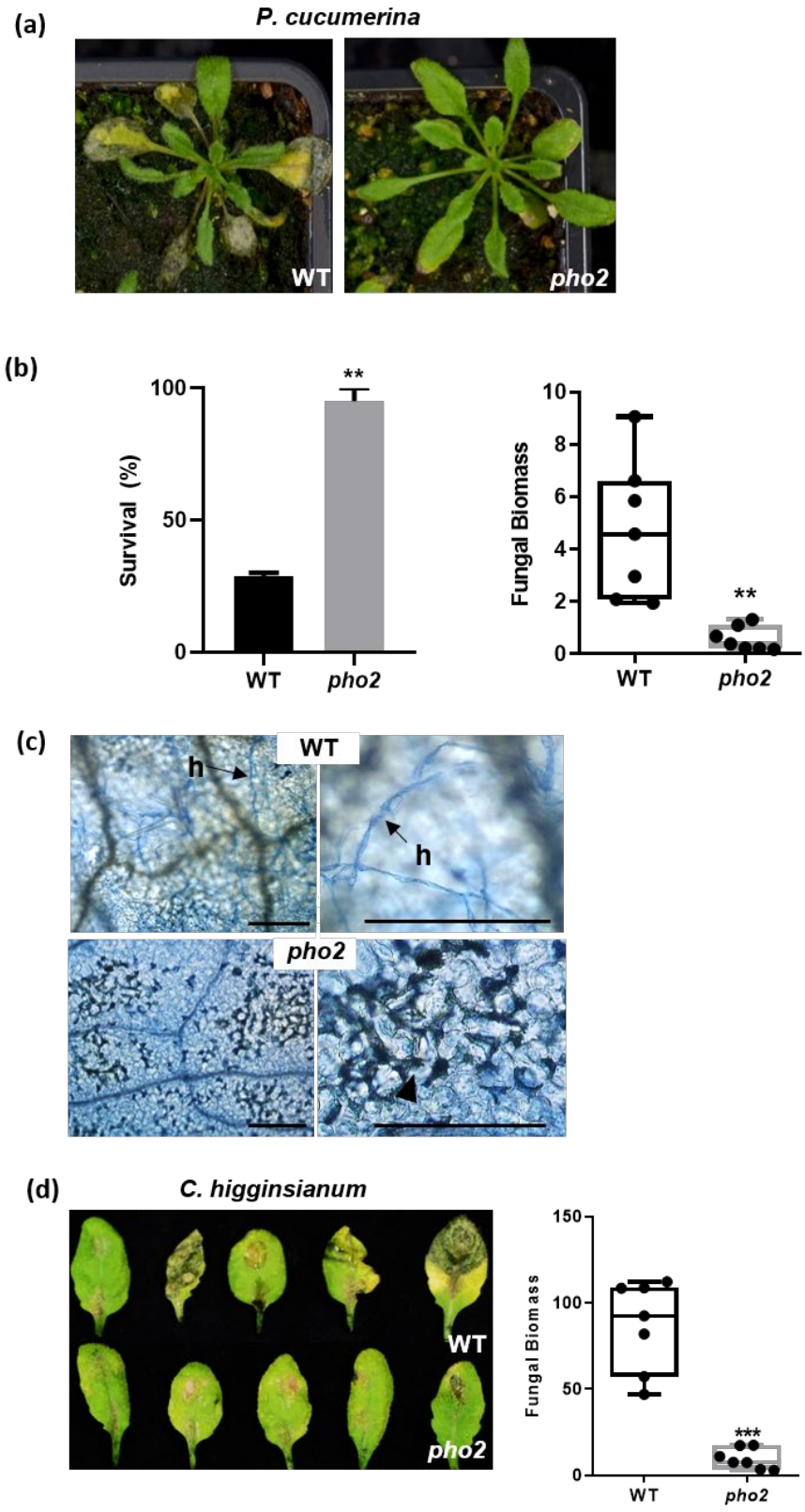

Figure 3. Resistance to infection by fungal pathogens in *pho2* mutant plants.
Three week-old mutant plants were inoculated with fungal *P. cucumerina* spores. Three independent experiments were carried out with similar results with at least 12 plants per genotype. **(a)** Disease phenotype of wild-type and pho2 plants upon inoculation with *P. cucumerina* spores (5 x 10^5spores/ml). Pictures were taken at 7 days post inoculation (dpi). **(b)**

Survival ratio of WT and *pho2* plants at 7dpi (left panel). Quantification of *P. cucumerina* DNA was carried out using specific primers of *P. cucumerina β-tubulin* at 7 dpi (right panel). Values are fungal DNA levels normalized against the Arabidopsis *UBIQUITIN21* gene (At5g25760). Data are mean ± SEM (n = 7) (*t* test, $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$). **(c)** Trypan blue staining of P. *cucumerina*-infected leaves and visualization of cell death and fungal growth. h, hyphae. Arrows and arrowheads indicate fungal hyphae and cell death, respectively. Bars represent 200 µm. (d) Disease phenotype of wild-type and pho2 plants at 8 days after inoculation with *C. higginsianum* spores (4 x 10^6 spores/ml).

Taken together, results here presented support that both *pho2* and miR399 overexpressing plants accumulate Pi in leaves and exhibit resistance to infection by fungal pathogens with a necrotrophic or hemibiotrophic lifestyle.

Stimulation of ROS production in Arabidopsis plants containing increased levels of Pi

One of the hallmarks of host–pathogen interactions is the overproduction of ROS as a plant defense mechanism, the so-called oxidative burst. ROS include various forms of reactive molecules, such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) or hydrogen peroxide (H_2O_2). Of them, H_2O_2, might act as both as signaling molecule for the activation of plant immune responses as well as an antimicrobial agent (Torres, Jones and Dangl, 2006). *RBOHD* (*Respiratory Burst Oxidase Homolog D*), a member of the Arabidopsis NADPH (Nicotinamide Adenine Dinucleotide Phosphate) oxidase gene family, has been shown to be responsible for ROS production after pathogen infection (Torres, Dangl and Jones, 2002; Kadota, Shirasu and Zipfel, 2015). Furthermore, ROS may promote cell death and limitation of pathogen spread.

Knowing that miR399 OE and *pho2* plants exhibit a pattern of cell death upon pathogen infection, we examined ROS accumulation in miR399 OE and *pho2* plants, both in the presence and in the absence of pathogen infection. Histochemical detection of H_2O_2 was carried out using the fluorescent probe H_2DCFDA (2', 7' dichlorofluorescein diacetate). H_2DCFDA was previously shown to detect different forms of ROS, mainly H_2O_2 but also hydroxyl radical and superoxide anion (Fichman, Miller and Mittler, 2019). Compared with wild-type plants, ROS accumulation could be observed in miR399 OE and *pho2* plants in the absence of pathogen infection (**Figure 4a**, upper panels). *P. cucumerina* infection further increased ROS levels in miR399 OE and *pho2* plants (**Figure 4a**, lower

panels), and at to a greater extent than in wild-type plants. Discrete regions accumulating ROS, most probably, correspond to infection sites. Similar results were observed by 3,3'-diaminobenzidine (DAB) staining of *P. cucumerina*-infected leaves (**Figure S3**). Thus, in the absence of pathogen infection, a higher level of ROS accumulation was observed in leaves miR399OE and *pho2* plants compared to WT, and its ROS level were further increased in response to inoculation with *P. cucumerina* spores (**Figure S3**).

In concordance with results obtained by histochemical localization of ROS, in the absence of pathogen infection, miR399 OE and *pho2* mutant plants exhibited an increase in RBOHD expression compared with wild type plants, although this difference was not statistically significant (**Figure 4b**). *P. cucumerina* infection was accompanied by an increase in *RBOHD* expression in all the genotypes (WT, miR399 OE and *pho2* plants), its expression reaching higher levels in fungal-infected miR399 OE and *pho2* plants than in fungal-infected wild-type plants (**Figure 4b**). Sustained overactivation of *RBOHD* might well account for the substantial increase in ROS accumulation that occurs in miR399 and *pho2* plants during pathogen infection. However, the possibility that other yet unknown mechanisms might contribute to ROS accumulation in these plants should not be discarded.

The observation that both miR399 OE and *pho2* plants accumulated Pi and had increased ROS production, these plants also exhibiting enhanced disease resistance (**Figure 1 and 2**), prompted us to investigate whether ROS production and disease resistance is affected by Pi supply in Arabidopsis. To address this question, wild-type plants were grown *in vitro* on media at different Pi concentrations (0.05 mM, 0.1 mM, 0.25 mM Pi, hereinafter referred as $P_{0.05}$, $P_{0.1}$ and $P_{0.25}$ plants). As expected, measurement of Pi content confirmed that increasing Pi supply to plants results in higher leaf Pi content (**Figure 4c**). To note, increasing Pi supply was accompanied by an increase in ROS accumulation (**Figure 4d**). Most importantly, upon pathogen inoculation, high-Pi plants consistently displayed enhanced resistance to infection by either *P. cucumerina* or *C. higginsianum* (**Figure 4e, f**, respectively). These observations support that high Pi supply fosters ROS production in Arabidopsis.

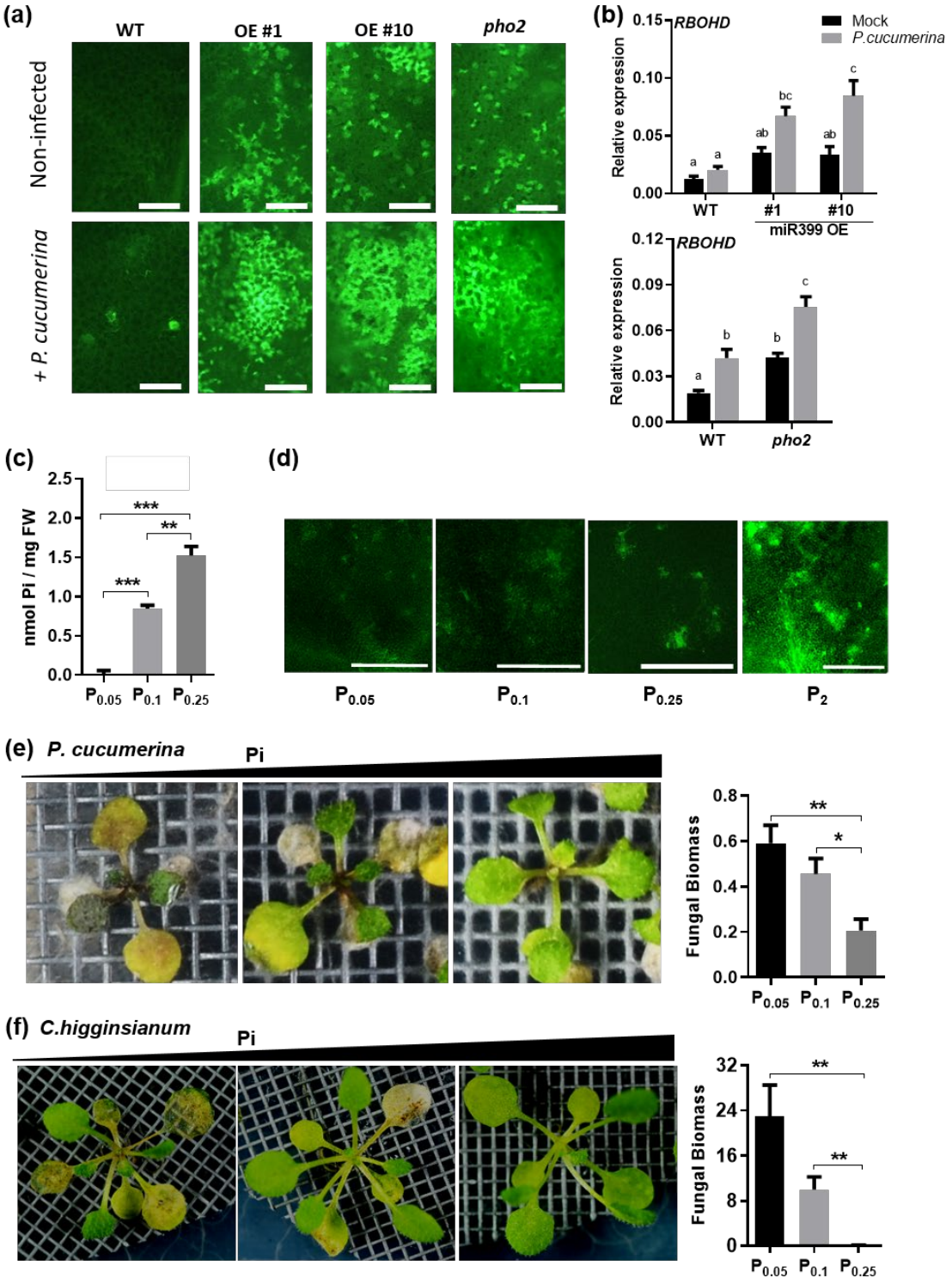

Figure 4. Enhanced accumulation of ROS and disease resistance in Arabidopsis plants that overaccumulate Pi.

(a) *In situ* histochemical detection of ROS in leaves of miR399 OE and *pho2* plants. Plants were spray-inoculated with *P. cucumerina* spores (5 x 10^5spores/ml), or mock-inoculated. Visualization of ROS accumulation was carried out using the fluorescent probe H2DCFDA at 2 days post inoculation (dpi). Bars represent 200 µm. **(b)** Expression of RBOHD in mock-inoculated and *P. cucumerina*-inoculated plants at 24hpi (black and grey bars, respectively). The expression values were normalized to the Arabidopsis *β-tubulin2* gene (At5g62690). Different letters represent statistically significant differences (ANOVA, HSD Tukey's test; $p \leq 0.05$). **(c)** Free Pi

content in plants that have been grown under different conditions of Pi supply. Plants were grown on agar plates for 1 week, transferred to fresh agar plates with medium containing different concentrations of Pi (0.05mM, 0.1mM, or 0.25mM). Plants were allowed to continue growth for one more week and then inoculated with fungal spores. Pi content was determined at the time of inoculation with fungal spores. **(d**) ROS accumulation in wild-type Arabidopsis (Col-0) plants that have been grown under different Pi supply conditions that is 0.05mM, 0.1mM, 0.25mM, or 2 mM Pi (P0.05, P0.1, P0.25, and P2, respectively). ROS was detected using H2DCFDA. Representative images are shown. Bars correspond to 1 mm. **(e)** Resistance to infection by *P. cucumerina* in Pi-treated Arabidopsis plants. Appearance of plants at 7 days post-inoculation (dpi) with *P. cucumerina* spores (4×10^6 spores/ml; left panel). Representative results from one of three independent infection experiments that gave similar results are shown. Right panel, fungal biomass determined at 3 dpi by quantitative PCR analysis using specific primers of *P. cucumerina* β-tubulin and normalized to Arabidopsis *UBIQUITIN21* gene (At5g25760). **(f)** Resistance to infection by *C. higginsianum* in Pi-treated Arabidopsis plants. Disease symptoms of Arabidopsis plants at 12 days post-inoculation with *C. higginsianum* spores (5×10^5 spores/ml). Right panel, fungal biomass determined at 7 dpi by quantitative PCR analysis using specific primers of *C. higginsianum ITS2* (*Internally transcribed spacer 2*). Means of three biological replicates, each one from a pool of at least three plants are shown in e and f (right panels; *t* test, $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$).

Pi-induced resistance to pathogen infection in Arabidopsis occurs through modulation of SA- and JA-dependent defense pathways

As previously mentioned, SA and JA play a critical role in the transcriptional reprograming of Arabidopsis plants in response to pathogen infection (Pieterse *et al.*, 2012). A pathogen-induced accumulation of ROS is also required for the induction of SA-dependent defenses, indicating that ROS and SA are intertwined in a complex regulatory network (Wang *et al.*, 2014; Herrera-Vásquez *et al*., 2015). To get deeper insights into the resistance phenotype of miR399 OE and *pho2*), we investigated the expression of defense genes linked to the SA and the JA pathways in these plants. The marker genes of the SA-mediated defense response here examined were: *PR1* (*Pathogenesis-Related 1*), *NPR1* (*Non-expressor of Pathogenesis-Related genes 1*), and *PAD4* (*Phytoalexin Deficient4*) (Jirage *et al.*, 1999). We found that, in the absence of pathogen infection, the expression of *PR1*, *NPR1* and *PAD4* was higher in both miR399 OE and *pho2* plants compared with wild-type plants, their expression further increasing during *P. cucumerina* infection in all the genotypes (**Figure 5a**).

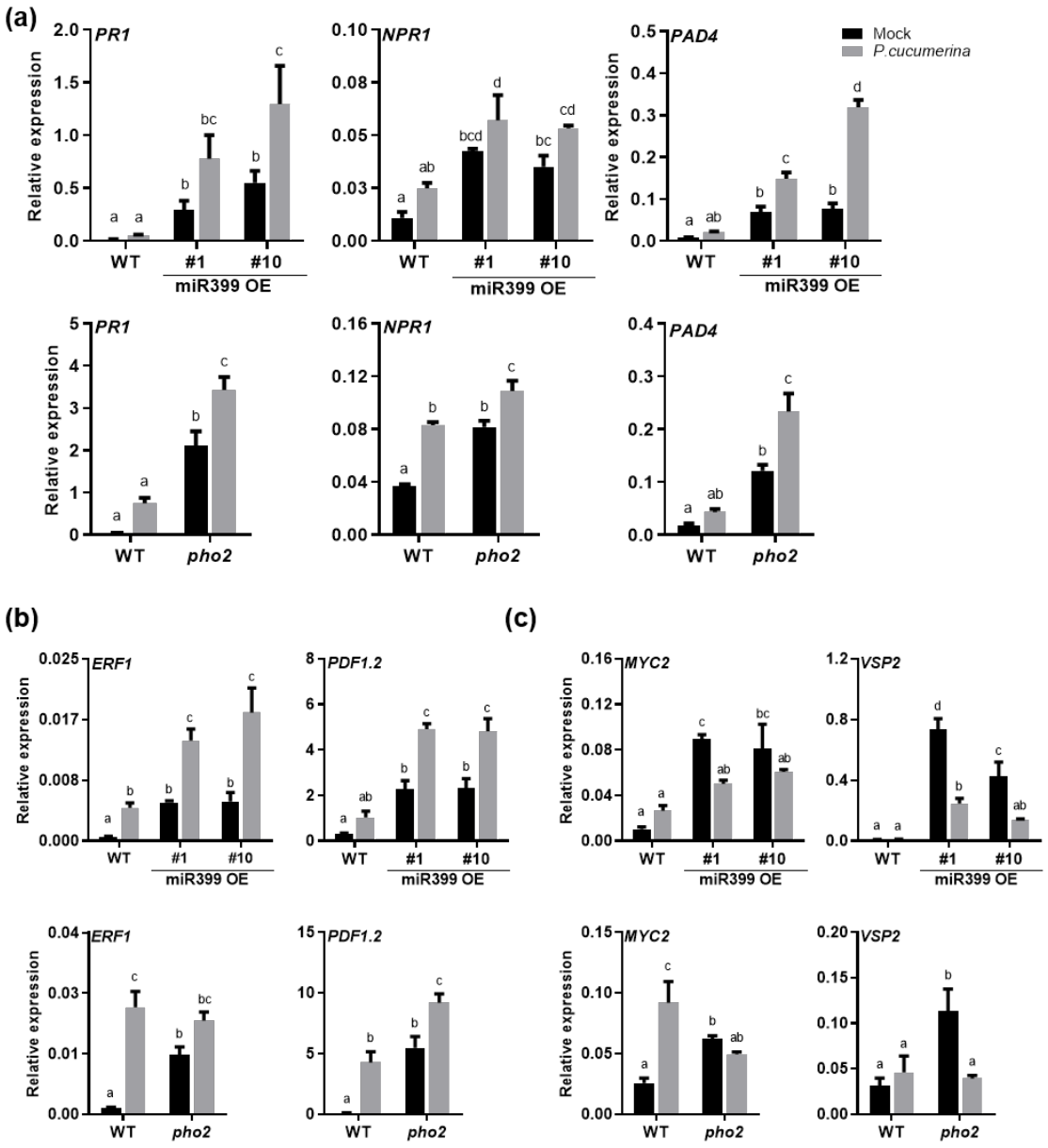

Figure 5. Expression of defense genes in miR399 OE and *pho2* mutant plants. Transcript levels were determined by RT-qPCR in mock-inoculated or *P. cucumerina*-inoculated plants at 24 hours post-inoculation (black and grey bars, respectively). The expression values were normalized to the Arabidopsis *β-tubulin2* gene (At5g62690). Three biological replicates (with 12 plants per genotype) were examined, with similar results. **(a)** Expression of genes involved in the SA pathway (*PR1, NPR1, PAD4*). **(b)** Expression of genes in the ERF branch of the JA pathway (*ERF1*, *PDF1.2*). **(c)** Expression of genes in the MYC branch of the JA pathway (*MYC2*, *VSP2*). Different letters represent statistically significant differences (ANOVA, HSD Tukey's test; $P < 0.05$).

Regarding the JA pathway, two branches are documented in Arabidopsis: the MYC2 branch, which is regulated by AtMYC2 (a basic helix-loop-helix-leucine zipper transcription factor), and the ERF branch, which is regulated by AtERF1 (a member of the APETALA/ERF transcription factor family) (Lorenzo *et al.*, 2004). PDF1.2 (*Plant*

Defensin 1.2) is commonly used as marker of the ERF branch, whereas the MYC branch is marked by the induction of *VSP2* (*Vegetative Storage Protein 2*) (Lorenzo *et al.*, 2004; Pieterse *et al.*, 2012; Wasternack and Hause, 2013; Zhang *et al.*, 2017). The ERF branch and the MYC branch of the JA signaling pathway have been reported to repress each other (Lorenzo *et al.*, 2004; Wasternack and Hause, 2013; Aerts *et al*., 2021).

We found that, in the absence of pathogen infection, the miR399 OE and *pho2* plants show up-regulation of transcription factor and defense marker genes associated to the two branches of the JA signaling pathway, namely the ERF1/PDF1.2 and MYC2/VSP2 branches) (**Figure 5b**). Pathogen infection further induced *ERF1* and *PDF1* expression in all the genotypes (wild-type, miR399 OE and *pho2* plants) (**Figure 5b**). In contrast, while *MYC2* and *VSP2* showed inducible expression by pathogen infection in wild-type plants, their expression was reduced in *P. cucumerina*-infected miR399OE and *pho2* plants (compared with the corresponding non-infected plants), a response that is more evident on *VSP2* expression (**Figure 5b**). Together, these findings support that resistance to pathogen infection in plants accumulating Pi (i.e. miR399 OE, *pho2* plants) is associated with a basal expression of SA- and JA-regulated genes (ERF and MYC2 branches). A pathogen-induced superactivation of genes involved in the SA- and ERF1 branch of the JA-defense pathway occurs in these plants, while the MYC branch of the JA signaling pathway is repressed during pathogen infection.

Further, we measured levels of the phytohormones SA and JA in miR399 OE and *pho2* plants. As shown in **Figure 6a** (upper panels), in the absence of infection, the SA level of miR399 OE and *pho2* plants was higher in both miR399 OE and *pho2* plants compared to wild-type plants. Upon pathogen infection, however, there were no significant differences in SA levels among wild-type, miR399 OE and *pho2* plants. We also noticed that the SA glucoside SAG (the storage from SA) accumulated at a higher level in the fungal-infected miR399OE and *pho2* plants relative to the fungal-infected wild-type plants, pointing to a strict control in the SA level in miR399OE and *pho2* plants through SAG accumulation.

Compared with wild type plants, miR399 overexpressor and *pho2* plants accumulated higher levels of JA, under non-infection and infection conditions (**Figure 6a**, lower

panels). Regarding the JA biosynthetic precursor 12-oxophytodienoic acid (OPDA), no significant differences were observed between wild type, miR399 OE and *pho 2* plants in the absence of pathogen infection, their level further increasing during infection. Taken together, SA and JA measurements in miR399 OE and *pho2* plants correlated well with the expression pattern of SA- and JA-responsive defense genes in miR399 OE and *pho2* plants (see **Figure 5**).

Additionally, we investigated whether Pi treatment modulates SA and JA signaling pathways. This study revealed that an increase in Pi supply results in increased SA, SAG, JA and OPDA levels (**Figure 6b**), which is consistent with up-regulation of SA- and JA-regulated defense genes (*PR1*, *NPR1* and *PDF1.2*; **Figure S4**).

Collectively, these results support that miR399 overexpression, loss-of-function of pho2, and Pi treatment enhanced accumulation of SA- and JA in leaves of Arabidopsis plants and up-regulation of SA- and JA-dependent defense responses. A Pi-mediated modulation of SA- and JA signaling pathways might well explain the phenotype of disease resistance that is observed in Arabidopsis plants that overaccumulate Pi, namely miR399 OE, *pho2* and wild-type plants grown under high Pi supply.

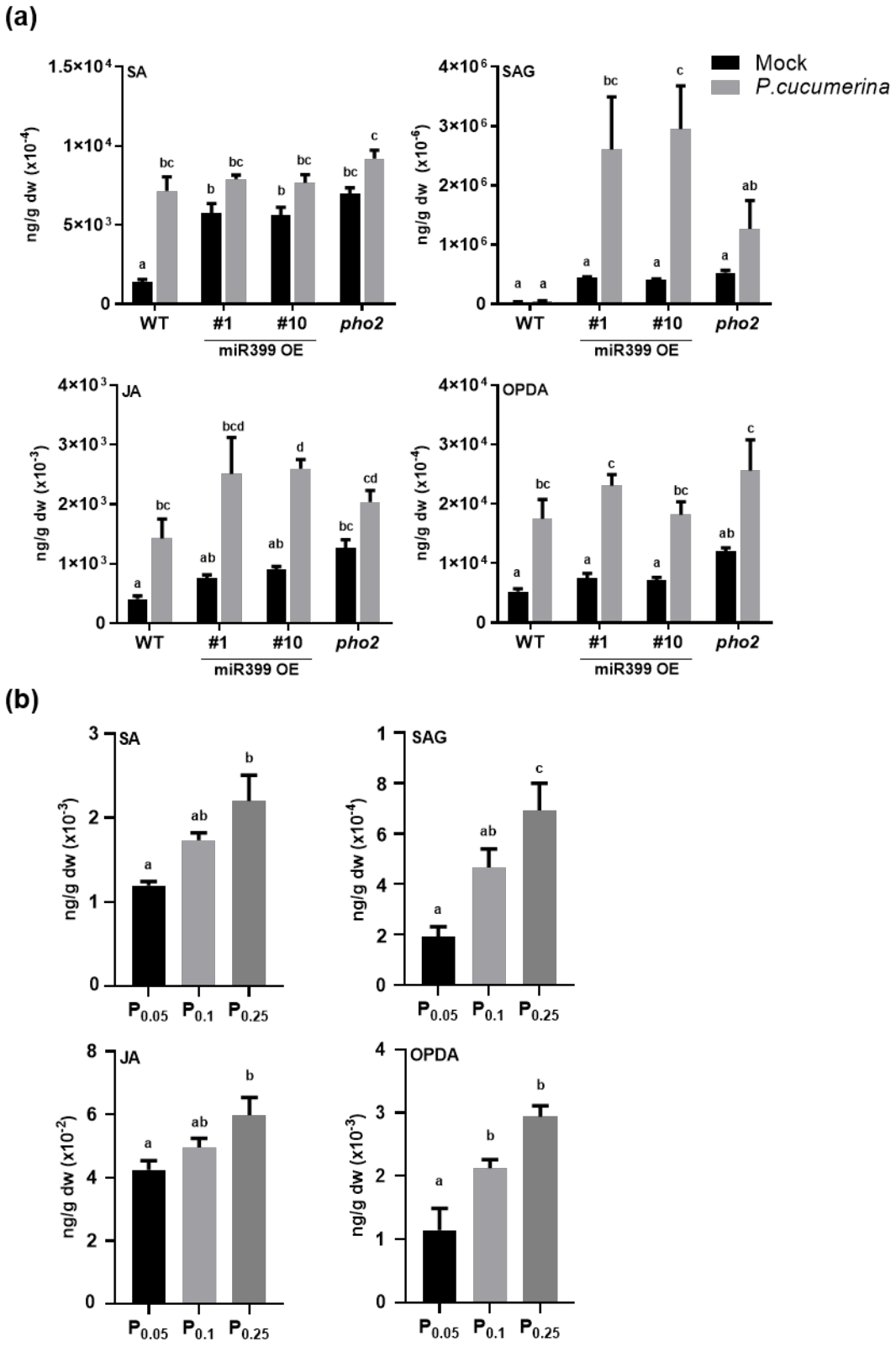

Figure 6. Enhanced levels of SA, SAG, JA and OPDA in leaves of Arabidopsis plants that overaccumulate Pi (miR399 OE, *pho2*, and wild-type plants that have been grown under different Pi supply conditions). **(a)** Hormone levels were measured in mock-inoculated and *P. cucumerina*-inoculated miR399 and *pho2* plants at 48hpi. **(b)** Hormone levels in wild-type plants that have been grown at the indicated Pi supply conditions (0.05, 0.1, and 0.25 mM Pi). Plants

were grown as indicated in Figure 4. Bars represent mean ± SEM. Different letters represent statistically significant differences (ANOVA, HSD Tukey's test; $P < 0.05$).

DISCUSSION

Nutrients play crucial roles in normal plant growth and development, and nutrient imbalance might also have a substantial impact on the predisposition of plants to resist pathogen attack. Depending on the identity of the interacting partners, nutritional imbalances caused by either nutrient excess or deficiency may determine the outcome of the interaction, resistance or susceptibility (Veresoglou *et al.*, 2013). On the other hand, although miRNA-mediated regulation of gene expression in processes involved in either nutrient stress or immune responses is well documented, less effort has been made to investigate miRNA function in crosstalk between pathogen-induced and nutrient pathways, in particular Pi.

In this study, we provide evidence that increasing Pi content in Arabidopsis results in enhanced disease resistance. Several pieces of evidence support this conclusion. On the one hand, we show that Pi accumulation caused by miR399 overexpression, loss-of-function of *PHO2*, or treatment with Pi, confers resistance to infection by fungal pathogens with necrotrophic (*P. cucumerina*) and hemibiotrophic (*C. higginsianum*) lifestyle. On the other hand, resistance in Arabidopsis plants overaccumulating Pi correlated with a basal expression of immune responses in the absence of pathogen infection, and stronger induction of defense responses during pathogen infection. Altogether, these findings support that miR399 functions as a positive regulator of Arabidopsis immunity, and reinforce the notion that miR399 plays a dual role in plants by controlling Pi homeostasis and immune responses.

Another finding of this study is that, under non-infection conditions, overaccumulation of Pi in Arabidopsis leaves is accompanied by ROS production, its level further increasing during pathogen infection. ROS accumulation correlated well with the expression pattern of *ROBHD* (encoding the NADPH oxidase responsible for the pathogen-induced production of ROS), which might explain, at least in part, the observed ROS accumulation in miR399 OE and *pho2* mutant plants. Hence, ROS accumulation in miR399OE and *pho2* plants might be responsible for the pattern of cell death observed in these plants during

pathogen infection, a response that is reminiscent of the pathogen-induced HR. In contrast, the fungal-infected wild-type plants did not exhibit cell death and showed extensive fungal growth. Additionally, elevated levels of ROS might contribute to the activation of immune responses in high Pi plants leading to a phenotype of disease resistance. From this point of view, it might be interesting to determine whether Pi-induced ROS accumulation can be generalized to other plant species.

Results here presented indicated that miR399OE and *pho2* plants accumulated 3-4 times more Pi than WT plants at the time of inoculation with fungal spores. By this developmental stage, plant growth is not compromised in plants accumulating this level of Pi. It is tempting to hypothesize that this increase in Pi content and/or ROS level is perceived by the host plant as a stressful situation, and that the plant responds to these signals with the induction of defense gene expression. An increase in Pi content might eventually increase intracellular and extracellular Pi levels for an increase in ATP level. Here, it should be mentioned that ATP has been shown to function as a DAMP signal after release into the extracellular space upon cellular damage, and that extracellular ATP enhances plant defense against pathogens through the activation of JA (Tanaka *et al.*, 2014b; Tripathi *et al.*, 2018). Further studies are needed to establish whether ATP levels are altered in high-Pi plants.

Gene expression analysis revealed regulation of the SA- and JA-defense signaling pathways in Arabidopsis plants overaccumulating Pi in leaves. Compared to wild type plants, all the high Pi plants here examined (miR399 OE, *pho2,* and plants grown under high Pi supply conditions) showed activation of SA-regulated (*PR1*, *NPR1, PAD4*) and JA-regulated (*ERF1* and *PDF1.2*; *MY2* and *VSP2*) genes under non-infection conditions. During pathogen infection, these genes also reached a higher level of expression in high Pi plants compared to wild type plants. SA and JA were reported to play a positive role in the regulation of resistance to *P. cucumerina* infection in Arabidopsis (Berrocal-Lobo *et al.*, 2002; Sánchez-Vallet *et al.*, 2012). Previous studies revealed that *PDF1.2* induction was associated to resistance to infection by necrotrophic fungi, including *P. cucumerina*, in Arabidopsis (Thomma *et al.*, 1998; Berrocal-Lobo *et al.*, 2002). Consistent with up-regulation of genes involved in the SA and JA signaling pathways, SA and JA accumulated in high Pi plants. In other studies, a feed-forward loop between SA and ROS production

(e.g. H_2O_2) has been reported in which ROS are involved both upstream and downstream of SA in the plant defense response to pathogen infection (Herrera-Vásquez *et al.*, 2015). We hypothesize that Pi content and, possibly also ROS accumulation, might influence defense hormone signaling.

Interestingly, although genes in the ERF1 and MYC2 branches showed higher expression in high Pi plants in the absence of pathogen infection, a different regulation in the two branches of the JA pathway was observed in high-Pi plants during pathogen infection. Whereas *ERF1* and *PDF1.2* expression is further increased during infection in high Pi plants (infected vs non-infected plants, each genotype), *MYC2* and *VSP2* expression diminished in these plants (infected vs non-infected plants, each genotype). We envisage that this differential regulation might be due to still unknown factors that cooperate in an antagonistic manner in the regulation of the ERF1 and MYC2 branches of the JA pathway during pathogen infection. In line with this, a negative correlation between the ERF1 and MYC2 branches has been previously described in the Arabidopsis response to different attackers such as pathogens and phytophagous insects (Lorenzo *et al.*, 2004; Pieterse *et al.*, 2012; Wasternack and Hause, 2013; L. Zhang *et al.*, 2017). Here, necrotrophic pathogens preferentially activate the ERF branch, while the MYC2 branch is activated by insect herbivory and wounding. A specialization in the host plant for modulation of each branch of the JA signaling pathway might then exist. It will be of interest to explore whether Pi accumulation has an effect on plant/insect interactions in Arabidopsis.

In plant-pathogen interactions, cross-talk between defense-related hormone pathways provides the plant with a powerful regulatory potential to control defense responses (Zheng *et al.*, 2012; Aerts *et al*., 2021). However, the type of induced response that is effective for disease resistance appears to vary depending on the host plant and pathogen identity. Although there are exceptions, pathogens with a biotrophic or hemibiotrophic lifestyle (such as *Pseudomonas syringae*) are generally more sensitive to SA-dependent responses, whereas necrotrophic pathogens are commonly deterred by JA/ET-dependent defenses (Glazebrook, 2005). The observation that high Pi plants (e.g. miR399 OE, *pho2*, and plants grown under high Pi supply conditions) show enhanced resistance to necrotrophic (*P. cucumerina*) and hemibiotrophic (*C. higginsianum*) fungal

pathogens makes it unlikely that the pathogen lifestyle determines disease resistance in high Pi plants.

Based on the results obtained in this study, a model is proposed to describe possible mechanisms underlying disease resistance in high Pi Arabidopsis plants (**Figure 7**). According to our model, miR399 overexpression and loss-of-function of *PHO2*, as well as growing plants under high Pi supply, increases Pi accumulation. An increase in Pi level would induce ROS production, as well as SA and JA accumulation. A basal expression of SA- and JA-dependent defense responses under non-infection conditions, in combination with a stronger induction of these responses during infection would allow the plant to mount an effective defense against pathogens.

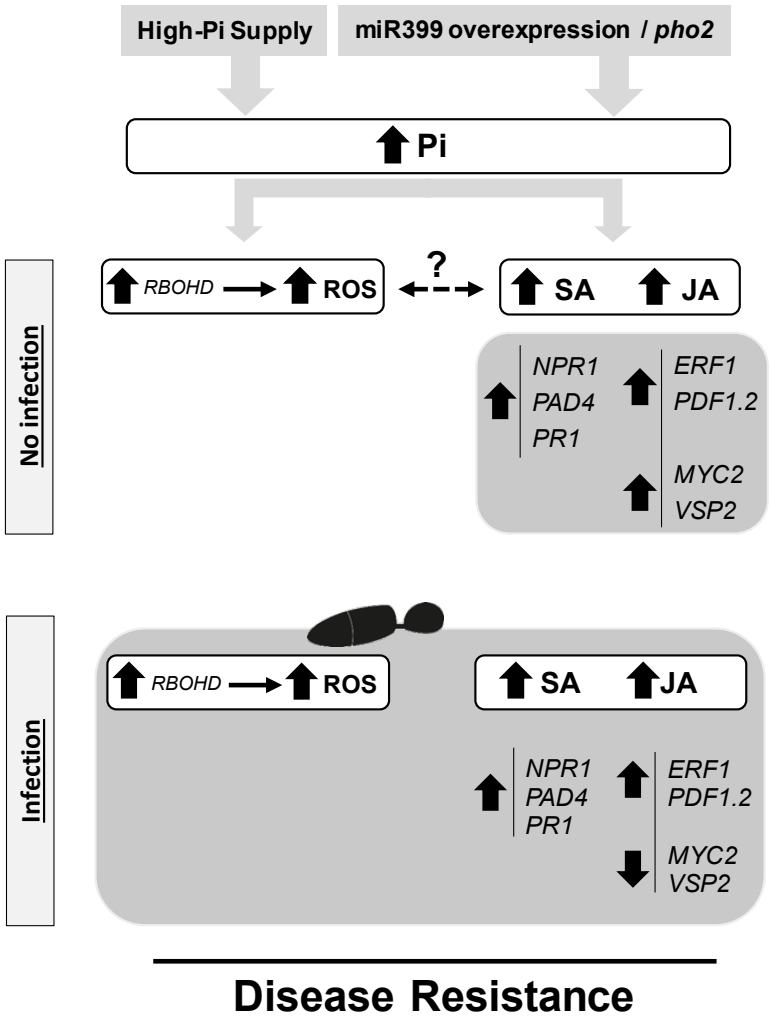

Figure 7. Proposed model to explain how Pi content and defense responses are integrated for modulation of resistance to infection by fungal pathogens in Arabidopsis.
Treatment with high Pi, *MIR399* overexpression and loss-of-function of *pho2* would trigger Pi accumulation which, in turn, would increase ROS and hormone (SA and JA) levels in Arabidopsis

leaves for the induction of genes involved in the SA- and JA-signaling pathway. Upon pathogen infection, the expression of genes involved SA and *ERF1/PDF1.2* branch of the JA pathway would be further induced, while genes in the *MYC2/VSP2* branch of the JA pathway are repressed. The interplay between Pi, ROS and hormone would allow the plant to mount an effective immune response. Although crosstalk between ROS and hormonal pathways have been described (Herrera-Vásquez, Salinas and Holuigue, 2015; Xia *et al.*, 2015), the exact mechanisms by which Pi content modulates ROS production and hormone content, and how these signaling pathways cross-talk to each other, in the coordinated regulation of Pi homeostasis and immune responses deserves further investigation.

The existence of links between components of the phosphate starvation response and disease resistance has been previously described (Chan, Liao and Chiou, 2021). For instance, enhanced resistance to infection by the bacterial pathogen *P. syringae* DC3000 and the oomycete pathogen *Hyaloperonospora arabidopsidis* was reported in *phr1* mutant plants, *PHR1* being the master transcriptional regulator of PSR in Arabidopsis (Castrillo, Teixeira, Paredes, *et al.*, 2017). This study also led the authors to propose that *PHR1* might fine-tune JA responses under Pi starvation in specific biological contexts, rather than being a regulator of the JA-signaling pathway (Castrillo, Teixeira, Paredes, *et al.*, 2017). In other studies, transgenic expression of a phytoplasma effector (SAP11) in Arabidopsis was found to trigger Pi starvation responses that are mainly dependent of *PHR1* (Lu *et al.*, 2014). The SAP11 transgenic plants overaccumulated Pi in leaves and were more susceptible to *P.syringae* pv.tomato DC3000 infection (Lu *et al.*, 2014). The PSR system also appears to control root colonization by the endophytic fungus *Colletotrichum tofieldiae* in Arabidopsis (Hiruma *et al.*, 2016; Frerigmann *et al.*, 2021). In citrus plants, the Pi content was associated to symptomology in the Huanglongbing (HBL) disease, where Pi deficiency favors development of HLB symptoms (Zhao *et al.*, 2013). In plant-insect interactions, Pi deficiency was found to induce the JA signaling pathway to enhance resistance to insect herbivory in a process that is partially under the control of *PHR1* (Khan *et al.*, 2016). Collectively, results here presented together with those found in the literature in other pathosystems support the existence of connections between Pi and immune signaling. These mechanisms should operate in a coordinated manner to properly balance nutrient responses and plant immunity.

Results from our study also raise intriguing questions about the impact of Pi content in disease resistance in different pathosystems. Thus, in this study we show that an

increase in Pi content positively regulates Arabidopsis immune responses which, in turn, enhances resistance to infection by fungal pathogens. Contrarily, in rice plants, a higher Pi content caused by miR399 overexpression or high Pi fertilization was found to negatively regulate defense gene expression, thus, increasing susceptibility to infection by the blast fungus *Magnaporthe oryzae* (Campos-Soriano *et al.*, 2020). In other studies, Pi deficiency was found to enhance resistance to *Verticillium dahliae* in cotton (Luo *et al.*, 2021) and insect herbivory in Arabidopsis (Khan *et al.*, 2016). Together, our results and those previously reported support that Pi content might positively or negatively regulate disease resistance depending on the interacting partners. It is tempting to speculate that different plants might have evolved diverse mechanisms to adapt to Pi alterations which, in turn, would determine a different effect of Pi in the regulation of immune responses. It is likely that integration of Pi stress (either deficiency or excess) and immune responses might vary depending on the host plant and the type of pathogen, the outcome of the interaction being also dependent on the role of the defense hormones SA and JA in that particular interaction. Alternatively, Pi might affect growth and/or pathogenicity of a fungal pathogen, either by creating a less favorable environment for pathogen growth, or by reducing the production of pathogen virulence factors. Clearly, these aspects deserve further investigation.

The fact that miR399 plays a positive role in regulating immune responses in Arabidopsis (present work), but a negative role in the regulation of immune responses in rice (Campos-Soriano *et al.*, 2020) is not unusual. Different miRNAs with an opposite role in resistance to pathogen infection have been previously described. For instance, miR398 overexpression compromises resistance against the bacterial pathogen *P. syringae* in Arabidopsis, while its overexpression in rice enhances resistance to the blast fungus *M. oryzae* (Li *et al.*, 2010; Y. Li *et al.*, 2019). These observations suggest that a particular miRNA might function either as a positive or as a negative regulator of immune responses depending on the host plant and the type of pathogen.

One of the major challenges plants face is defending against pathogen infection under continuous changes in nutrient availability, particularly Pi availability. Results here presented have set the basis for further research to elucidate the exact mechanisms by which Pi and pathogen-induced interact with each other in plants. Further studies,

however, should be conducted on a case-by-case basis in different plant/pathogen interactions. A better understanding of these mechanisms will allow the development of novel strategies to improve disease resistance in plants.

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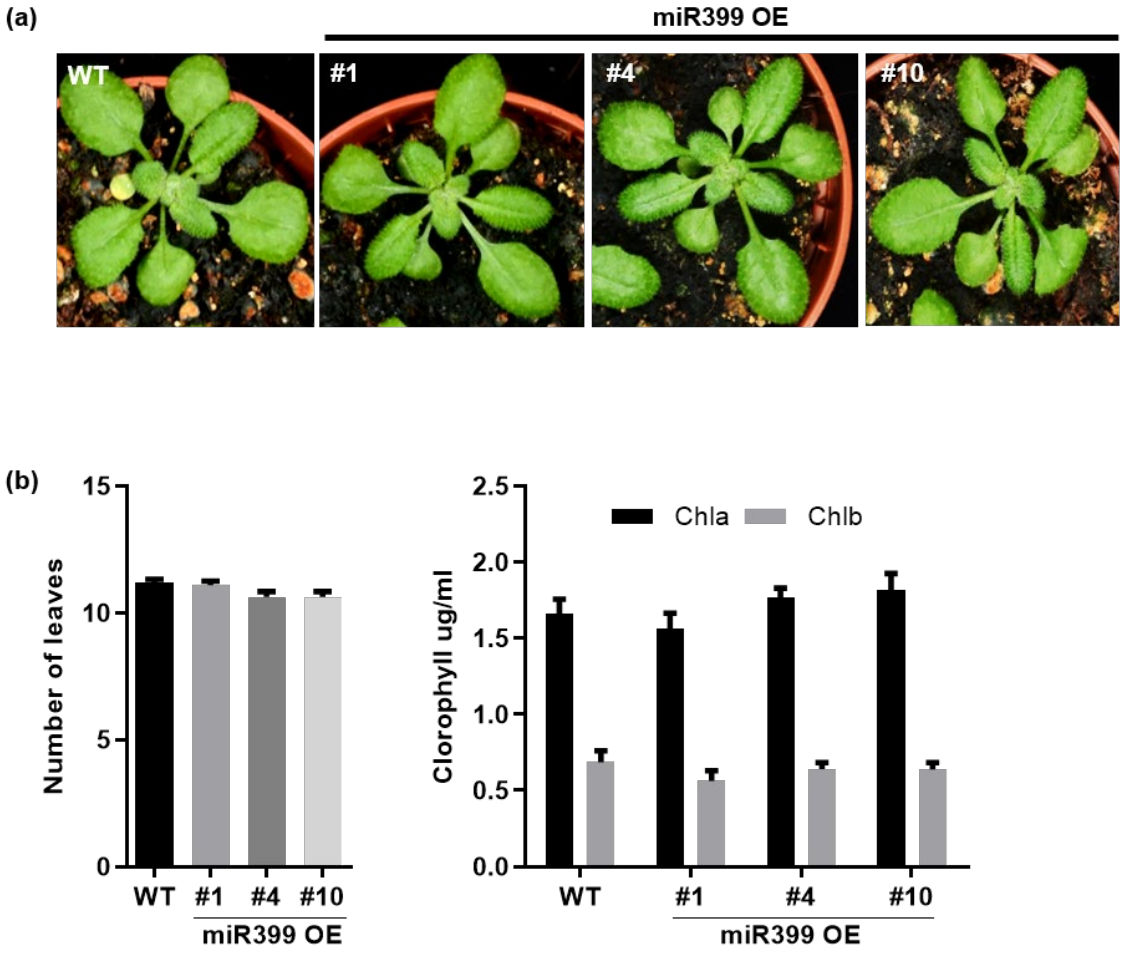
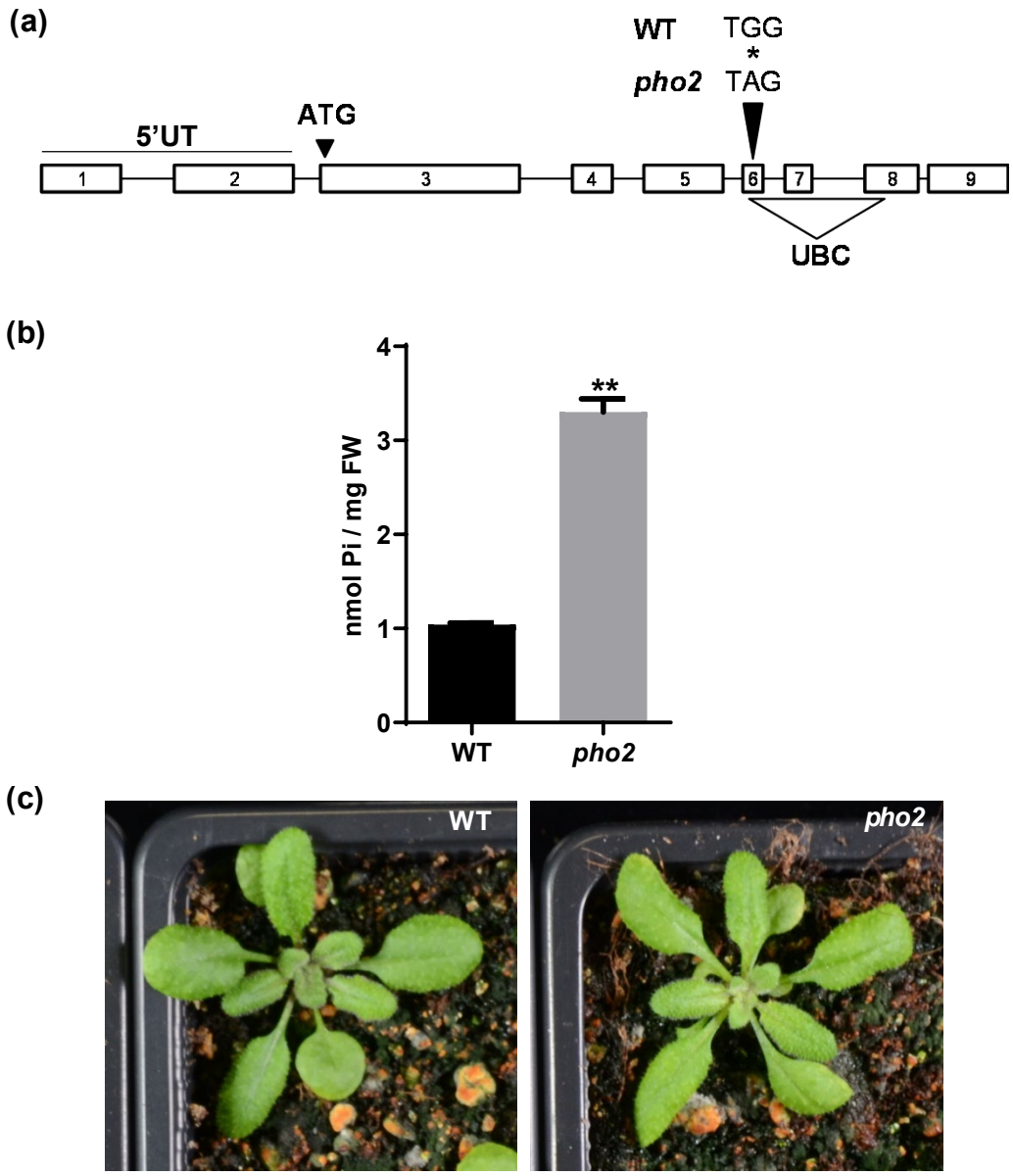
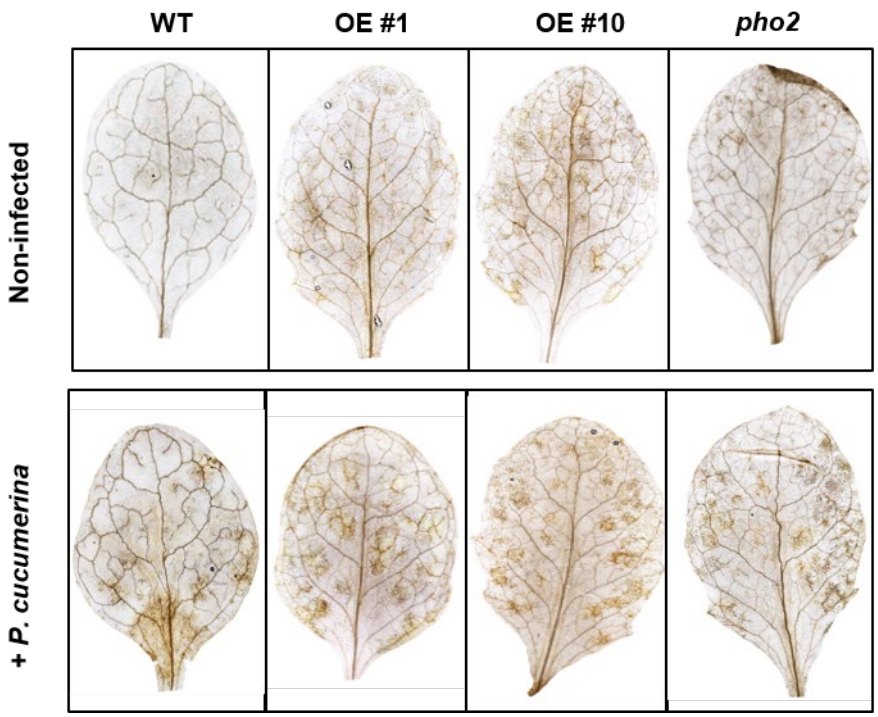
SUPPLEMENTAL MATERIAL



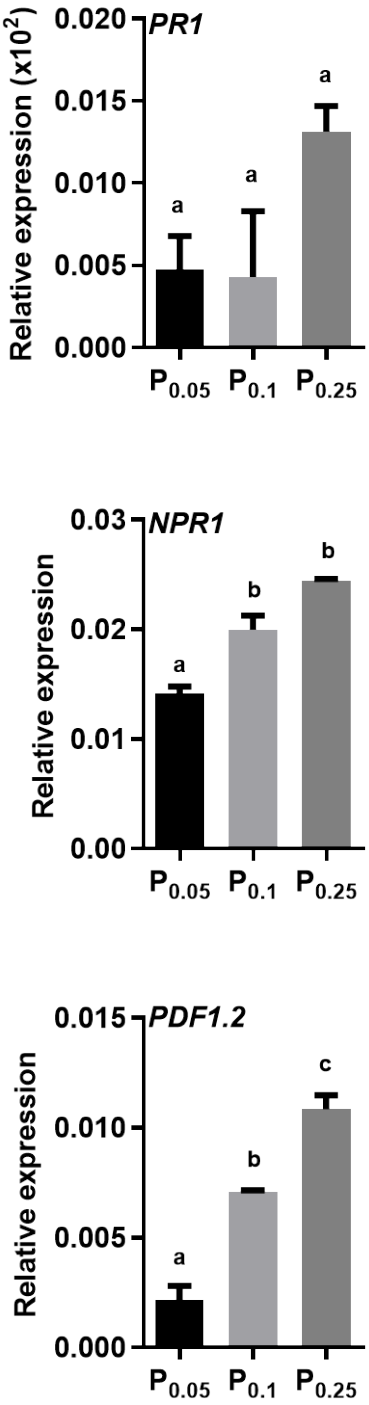
Supplemental **Figure S1**. Phenotypical analysis of miR399 OE plants.
Appearance **(a)**, and number of leaves and chlorophyll content of 3 week-old miR399 OE plants **(b)**.



Supplemental Figure S2. Analysis of *pho2* mutant plants under non-infection conditions.
(a) Representation of *pho2* mutation from the Arabidopsis Biological Resource Center (ABRC, ref. CS8508). The translation initiation site (ATG) and the ubiquitin conjugating domain (UBC) are indicated. The asterisk indicates the point mutation at exon 6 of *PHO2* causing an early termination. 5´UT, 5´untranslated region. **(b)** Pi content in leaves of *pho2* mutant plants. Bars represent mean ± *SEM* (n=3 and three plants per replicate) (*t* test, **p* ≤ 0.05, ***p* ≤ 0.01 and ****p* ≤ 0.001). **(c)** Appearance of three weeks old *pho2* mutant plants.



Supplemental Figure S3. *In situ* detection of ROS in leaves of miR399 OE and *pho2* plants by DAB staining. Plants were spray-inoculated with *P. cucumerina spores* (5 x 105spores/ml), or mock-inoculated. Visualization of H_2O_2 accumulation was carried out at 2 days post inoculation (dpi). Compared with wild-type plants, a basal accumulation of H_2O_2 was observed in leaves of non-inoculated miR399 OE and pho2 plants (upper panels). Upon pathogen challenge, discrete sites of DAB staining were evident in all the genotypes (wild type, miR399 OE and *pho2* plants) (lower panels).



Supplemental Figure S4. Expression of defense-related genes in wild-type Arabidopsis plants that have been grown under different Pi supply (0.05mM, 0.1mM, or 0.25mM). Different letters represent statistically significant differences (ANOVA, HSD Tukey's test; $P < 0.05$).

Supplemental table S1. List of oligonucleotides.

Gene name	Accession Number		Sequence (5'-3')
For expression analysis			
β-Tubulin 2	*At5g62690*	Fw	TGTTCAGGCGAGTGAGTGAG
		Rv	ATGTTGCTCTCCGCTTCTGT
Pre-miR399		Fw	TGCATAAATGTTTGTGGTGAGC
		Rv	GAATTACCGGGCAAATCTCCT
Pre-miR827		Fw	ACATGTTGATCATCCTTGTGTTGA
		Rv	CCAAGAAGCGATGCAAAACCA
miR399 stem loop		Rt	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCCGGGC
		Fw	GCGGTGCCAAAGGAGATTT
PHO2	*At2g33770*	Fw	AGGTTTGAAGCTCCACCCTCA
		Rv	CCCAAGATGTGATTGGAGTTCC
PR1	*At2g14610*	Fw	GATGTGCCAAAGTGAGGTGTAA
		Rv	GGCTTCTCGTTCACATAATTCC
NPR1	*At1g64280*	Fw	CCGGAAGAGCTTGTTAAAGAGA
		Rv	ATCCGAGTCAAGTGCCTTATGT
PAD4	*At3g52430*	Fw	CGAATACATTGGTGACGAAGAA
		Rv	ACCCATTTTGCACTTGAACTCT
ERF1	*At3g23240*	Fw	AACACTCGATGAGACGGAGAAT
		Rv	CTCCCAAATCCTCAAAGACAAC
PDF1.2	*At2g26020*	Fw	CAACAATGGTGGAAGCACAG
		Rv	CTTGCATGCATTGCTGTTTC
MYC2	*At1g32640*	Fw	ATTAATGACCCGATTGGAACAC
		Rv	TGAGCTACCGTTCTCAAACTGA
VSP2	*At5g24770*	Fw	CTCGTCGATTCGAAAACCAT
		Rv	TTCTGCAGTTGGCGTAGTTG
RBOHD	*At5g47910*	Fw	ATTACAAGCACCAAACCAG
		Rv	TTCTCCGACCATCTCACTA
For fungal DNA quantification			
P.cucumerina_tubulin		Fw	CAAGTATGTTCCCCGAGCCGT
		Rv	GAAGAGCTGACCGAAGGGACC
Ch_ITS2		Fw	AAAGGTAGTGGCGGACCCTC
		Rv	GGCAAGAGTCCCTCCGGAt
Ubiquitin21	*At5g25760*	Fw	AAAGGACCTTCGGAGACTCCTTACG
		Rv	GGTCAAGAATCGAACTTGAGGAGGTT

CHAPTER II

Nitrogen limitation Adaptation (*NLA*) regulates Pi accumulation and resistance to infection by fungal pathogens in Arabidopsis by modulating salicylic acid- and jasmonic acid-mediated signaling and camalexin accumulation

Results presented in this chapter are included within a manuscript currently in preparation.

ABSTRACT

In nature, plants have to cope with a suite of biotic and abiotic stresses which commonly occur simultaneously. Interactions between signaling pathways triggered a particular type of stress might, however, affect the plant response to another stress. In particular, nutrient stress appears to be an important factor in determining the disease resistance in plants. Both resistance and susceptibility have been observed in plants under nutrient excess or deficiency, a response that appears to be dependent on the plant species and the type of pathogen. Phosphorus is an essential nutrient for plant growth that might also impose stress to the plant which, in turn, might impair the defense response to pathogens. It is well known that, under Pi limiting conditions, plants activate a number of signaling pathways, the plant starvation response (PSR), that increases Pi uptake through the roots and contributes to maintenance Pi homeostasis in the plant. Among others, *NLA* (*Nitrogen limitation Adaptation*) and its regulatory miRNA, miR827, are key components of PSR. In this study, we investigated whether *NLA* and miR827 play a role in the defense response of Arabidopsis plants to infection by necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Colletotrichum higginsianum*) fungal pathogens. We show that pathogen infection and treatment with fungal elicitors is accompanied by an increase in *MIR827* expression and down-regulation of *NLA*. Both, loss-of-function of *NLA* and *MIR827* overexpression causes an increase in phosphate (Pi) content which results in resistance to pathogen infection. Compared with wild-type plants, a higher callose deposition and ROS production occurs in *nla* plants, a response that is also observed in Pi-treated wild-type plants. The *nla* mutant plants also accumulated higher levels of the defense-related hormones salicylic acid (SA) and jasmonic acid (JA) in the absence of pathogen infection, which is consistent with a higher expression of SA- and JA-regulated defense genes in these plants. Upon pathogen challenge, SA and JA levels further increased in *nla* plants. We also show that the phytoalexin camalexin accumulated at a higher level in *nla* plants during pathogen attack. Together, these results support that *NLA* functions as a negative regulator of Arabidopsis immunity and that overaccumulation of Pi in *nla* plants positively affects resistance to infection by fungal pathogens. This piece of information reinforces the notion that cross-talk between phosphate signaling pathways and immune signaling pathways regulates disease resistance in Arabidopsis.

INTRODUCTION

Phosphorous (P) is one of the most important macronutrients required for plant growth and development. It is a key component of fundamental biomolecules, such as nucleic acids phospholipids, and ATP, the principal molecule for storing and transferring energy in cells. P is also a central regulator in numerous metabolic reactions that also functions in signaling pathways and modulation of protein activity. Plants absorb phosphorus from the soil in the form of inorganic form (Pi). Although P levels in soils are usually high, its bioavailability is often extremely low which represents a limiting factor for plant growth.

Plants have evolved different mechanisms to cope with Pi limitation. At the molecular level, plants develop adaptive mechanisms to enhance the acquisition of external Pi through the activity of phosphate transporters, collectively known as the Pi starvation response (PSR) (Yang and Finnegan, 2010; Puga *et al.*, 2017; Wang *et al.*, 2021). In *Arabidopsis thaliana*, the transcription factor PHR1 (PHOSPHATE STARVATION RESPONSE 1) is a central regulator of Pi starvation signaling leading to the induction of phosphate transporter genes of the *PHT1* family (Bustos *et al.*, 2010; Nilsson *et al.*, 2012; Guo *et al.*, 2015). *AtPHR1* regulates a number of Pi starvation-induced genes through binding a P1BS (PHR1 specific binding sequence) *cis*-element, which is present in the promoter regions of Pi starvation-induced genes (Rubio *et al.*, 2001). PHR1 also induces the expression of two microRNAs (miRNAs), miR399 and miR827, which negatively regulate the expression of *PHO2* (*PHOSPHATE 2*, a ubiquitin E2 conjugase) and *NLA* (*NITROGEN LIMITATION ADAPTATION*, a ubiquitin E3 ligase), respectively (Delhaize and Randall, 1995; Lin *et al.*, 2013). PHO2 and NLA mediate the ubiquitination and degradation of the plasma-membrane-localized PHT1 transporters. In this way, miRNA-mediated down-regulation of *PHO2* and *NLA* relieves the negative regulation over PHT1 transporters, thus, increasing Pi uptake in the roots (Bari *et al.*, 2006; Kant *et al*., 2011). Whereas considerable progress has been made in characterizing the regulatory mechanisms that underlie adaptation of plants to Pi starvation, less is known about adaptive mechanisms in plants under Pi excess condition.

In nature, plants are simultaneously exposed to different environmental stresses, biotic and abiotic stresses, but so far most research has concentrated on understanding plant responses to individual abiotic or biotic stresses. However, the simultaneous occurrence

of nutrient stress might affect disease resistance in plants, and cross-talk between plant responses induced by one stress may result in positive or negative impact over the other stress (Saijo and Loo, 2020). Stress caused by excess or deficiency of nutrients might affect disease resistance, as inferred by the observed effects of fertilization on the incidence of a specific disease, or the comparison of nutrient content in susceptible or resistant plants. As an example, high nitrogen or phosphate fertilization has been shown to increase susceptibility to infection with the blast fungus *M. oryzae* in rice (Ballini *et al.*, 2013; Campos-Soriano *et al.*, 2020). It is also true that the impact of nutrient stress (deficiency or excess) on disease resistance is difficult to predict, as different results are observed in the literature regarding the outcome of the interaction, resistance, susceptibility, depending on the identity of the host and the pathogen (Veresoglou *et al.*, 2013). Unfortunately, the molecular mechanisms involved in nutrient responses and innate immunity have been investigated separately from each other.

Plants have an innate immune system to defend themselves against pathogen infection. The plant immune system consists of interconnected processes that are induced upon recognition of the pathogen. Depending on the molecules that are recognized by the host plant, two immune systems have been defined. Plants recognize pathogen epitopes, known as Pathogen-Associated Molecular Patterns (PAMPs, also known as elicitors). This recognition activates a general defense response referred to as PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Boller and Felix, 2009; Li *et al.*, 2020). PTI components include reinforcement of cell wall and papillae formation, production of reactive oxygen species (ROS) and induction of defense-related genes (e.g. *Pathogenesis-Related* or *PR* genes). Pathogens adapted to their host have evolved effectors that are delivered to plant cells and suppress PTI leading to disease susceptibility. During co-evolution, plants have evolved another immune system in which pathogen effectors, or host proteins modified by these effectors, are recognized by proteins encoded by resistance (R) genes, the so called Effector-triggered Immunity (ETI). Whereas PTI contributes to resistance to diverse pathogens, ETI is pathogen strain or race specific and is often associated with programmed cell death, a response which is referred to as the hypersensitive response (HR).

Phytoalexins are low molecular weight antimicrobial compounds that take part in the defense system used by plants against pathogens (Ahuja *et al.*, 2012). The major phytoalexin involved in resistance against pathogens in Arabidopsis is camalexin, a sulfur-containing tryptophan-derived secondary metabolite. Camalexin accumulation has been correlated with resistance to necrotrophic fungi such as *Plectosphaerella cucumerina*, *Botrytis cinerea* and *Alternaria brassicicola* (Ferrari *et al.*, 2003; Nafisi *et al.*, 2007; Sanchez-Vallet *et al.*, 2010).

Phytohormones also play an essential role in the regulation of plant immune responses. In particular, the involvement of salicylic acid (SA), jasmonic acid (JA, and JA derivatives), and ethylene in the regulation of defense responses to pathogens in plants has long been recognized (Denancé *et al.*, 2013; Berens *et al.*, 2017; Aerts *et al.*, 2021). Synergistic and antagonistic interactions between hormone signaling pathways allow fine-tune responses to different pathogens.

Links between Pi starvation signaling and immune signaling have been described in Arabidopsis plants (Castrillo *et al.*, 2017; Chan *et al.*, 2021). For instance, *phr1* Arabidopsis mutants were found to be more resistant to infection by the bacterial pathogen *P. syringae* DC3000 and the oomycete pathogen *Hyaloperonospora arabidopsidis* (Castrillo *et al.*, 2017). Transcript profiling of *phr1* mutant plants indicated that PHR1 negatively regulates the immune response triggered by the bacterial elicitor peptide flagellin22 (flg22). This study also led the authors to propose that the Arabidopsis plants prioritizes nutritional status over defense (Castrillo *et al.*, 2017). Also in Arabidopsis, transgenic expression of a phytoplasma effector (SAP11) was found to trigger Pi starvation responses that are mainly dependent of *PHR1* (Lu *et al.*, 2014). The SAP11 transgenic plants overaccumulated Pi in leaves and were more susceptible to *P.syringae* pv.tomato DC3000 infection (Lu *et al.*, 2014).

Results presented in Chapter 1, demonstrated that MIR399 overexpression and loss-of-function of *PHO2,* the miR399 target gene, leads to an increase in Pi content in leaves of Arabidopsis plants. Up-regulation of genes involved in SA and JA signaling pathways occurs in miR399 OE and *pho2* plants, which is in agreement with the observation that these plants accumulated higher levels of SA and JA than wild-type plants. Both miR399

OE and *pho2* plants exhibited enhanced resistance to infection by necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Colletotrichum higginsianum*) fungal pathogens. Contrary to this, our group previously described that Pi accumulation caused by in miR399 overexpression in rice compromises defense mechanism, thus, resulting in a phenotype of susceptibility to infection by the rice blast fungus (Campos-Soriano *et al.*, 2020). This piece of information supports that Pi excess might have an effect on the expression of immune responses, and reinforces the idea of cross-talk between Pi signaling and immune signaling in plants. Increasing Pi content might, however, result in a different outcome of plant/pathogen interactions (e.g. resistance to infection by fungal pathogens in Arabidopsis and susceptibility to the rice blast fungus in rice).

In this study, we investigated whether the miR827/NLA module plays a role in disease resistance in Arabidopsis. As previously mentioned, miR827 down-regulates *NLA* expression in Pi-starved Arabidopsis plants (Hsieh *et al.*, 2009). *NLA* encodes a RING-type ubiquitin ligase responsible of ubiquitination of PHT1 (Kant *et al*., 2011). Even though the *NLA* gene was originally described as a positive regulator in the adaptive response of Arabidopsis plants to nitrogen (N) limitation (Peng *et al.*, 2007), it is widely recognized that *NLA* also plays a pivotal role in the regulation of Pi homeostasis in Arabidopsis (Lin *et al.*, 2013). Loss-of-function of *NLA* results in overaccumulation of PHT1 phosphate transporters, thereby increasing Pi uptake (Kant *et al*., 2011).

Results here presented demonstrated transcriptional activation of *MIR827*, and subsequent down-regulation of *NLA,* in response to treatment with fungal elicitors or SA. Both *nla* and miR827 overexpressor plants accumulated Pi in their leaves, these plants exhibiting resistance to infection by fungal pathogens with necrotrophic (*P. cucumerina* and hemibiotrophic (*C. higginsianum*) lifestyles. Upon pathogen infection, *nla* plants showed higher deposition of callose and ROS accumulation compared with wild-type plants. We also show that *nla* plants have stronger induction of SA- and JA-dependent defense genes and camalexin biosynthesis genes, which correlated well with the accumulation of SA, JA and camalexin. Overall, the results presented here support that miR827 functions as a positive regulator of Arabidopsis immunity.

EXPERIMENTAL PROCEDURES

Plant material infection assays and elicitors treatment

For infection or elicitor treatment experiments, *Arabidopsis thaliana* plants were grown in a mixture of soil:perlite:vermiculite (2:1:1) and modified Hoagland half strength medium, under neutral photoperiod (12h light / 12h dark), 60% of humidity and a temperature of 22ºC ± 2ºC for three weeks. The fungus *Plectosphaerella cucumerina* was grown on PDA (Potato Dextrose Agar) plates with chloramphenicol (34 µg/ml). *Colletotrichum higginsianum* was grown on Oatmeal agar plates in darkness. Fungal spores were collected by adding sterile water to the surface of the mycelium, and adjusted to the desired final concentration using a Bürker counting chamber. Plants were spray-inoculated with a spore suspension of *P. cucumerina* (5 x 10^5 spores/ml), or mock-inoculated. *C. higginsianum* was locally-inoculated with a spore suspension at 4 x 10^6 spores/ml (10 µl/leaf and 5 leaves/plant). Fungal-inoculated and mock-inoculated plants were maintained under high humidity and disease symptom development was followed with time. Lesion area was measured with software ImageJ (National Institute of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij/). Three independent experiments were performed with at least 12 plants per genotype in each experiment. Statistically significant differences were determined by t-test. For *in vitro* experiments, two-week old Arabidopsis plants were spray-inoculated with *P. cucumerina* (4 x 10^6 spores/ml). Fungal biomass was quantified by real-time PCR using specific primers for the corresponding fungus and the Arabidopsis *UBIQUITIN21* (At5g25760) gene as the internal control (Soto-Suárez *et al.*, 2017). PCR primers are listed in Table S1. Elicitor treatment was performed by spraying plants with an elicitor extract obtained from *P. cucumerina* (300 µg ml^{-1}) as described (Casacuberta *et al.*, 1992).

For Pi treatment experiments, plants were grown *in vitro* on meshes placed on agar plates with modified Hoagland half strength medium containing 0.25 mM KH_2PO_4 for one week. Seedlings were then transferred to fresh agar-medium at the desired concentration of Pi (0.05, 0.25, or 2 mM Pi). The plants were allowed to continue growing for one more week under each Pi regime. The *in vitro*-grown plants were then inoculated with a spore suspension of *P. cucumerina*.

Plant tissue staining

For trypan blue staining, leaves were fixed by vacuum infiltration for 1h in ethanol:formaldehyde:acetic acid (80:3.5:5 v/v), stained with lactophenol blue solution for one hour, and then washed with chloral hydrate for 15 minutes. Leaves were placed on glass slides with glycerol and observed using a Leica DM6 microscope under bright field.

Aniline blue staining was used to determine callose deposition. For this, chlorophyll was removed, with 70% ethanol, from leaves that were then incubated in 70 mM phosphate buffer (pH 9.0) supplemented with aniline blue (0.01% wt/vol) with vacuum for 30 min. Samples were maintained in dark conditions for 2 h. Leaves were observed with Leica DM6 microscope under UV illumination. Callose deposition was quantified by determining the relative number of callose-corresponding pixels relative to the total number of pixels using ImageJ software (Luna *et al.*, 2011).

For H_2DCFDA staining, the Arabidopsis leaves were placed on a solution of H_2DCFDA (at a concentration of 10µM), vacuum infiltrated during 5 minutes, and then maintained in darkness for 10 minutes. Two washes with distillated water were performed. Photographs were taken on a Leica DM6 microscope to visualize green fluorescence. DCFDA staining was quantified by determining the relative number of DCFDA-corresponding pixels relative to the total number of pixels using ImageJ software.

Generation of transgenic Arabidopsis Agrobacterium-mediated transformation of Arabidopsis and histochemical analysis of GUS activity

To obtain the *MIR827promoter:GUS* construct, the DNA sequence of the *MIR827* promoter region was extracted from the NCBI (http://www.ncbi.nlm.nih.gov). The transcription start site was identified by using the transcription start site identification program for plants (http://linux1.softberry.com/) and *cis* elements were determined using the PLACE database (https://www.dna.affrc.go.jp/PLACE). The DNA sequence covering 1.2 kb upstream of the transcription start site of *MIR827* was amplified by PCR from genomic DNA, and

cloned into the pKGWFS7 plant vector. The PCR product was verified by sequencing. The plant expression vector was transferred to the *Agrobacterium tumefaciens* strain GV3101. Arabidopsis (Col-0) plants were transformed using the floral dip method.

Analysis of GUS activity

Histochemical staining of GUS enzyme activity was performed according to Jefferson *et al.*, 1987. Leaves were fixed by vacuum infiltration for 1 h in ethanol : formaldehyde : acetic acid (80 : 3.5 : 5 by vol.), stained with lactophenol blue solution for 4 h and washed with 70% ethanol (5 min). Leaves were placed on glass slides with glycerol and observed using a Leica DM6 microscope. Hormone treatment with salicylic acid (SA) was performed in a SA solution of 100 µM during 12 hours and darkness.

Measurements of Pi

The Pi content of Arabidopsis plants was determined as previously described (Versaw and Harrison, 2002). For each experiment four biological replicates (of three plants/replicate) were used. Statistical *t* test analysis was used to analyze the data.

Gene expression analyses

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from DNAse-treated total RNA (1 µg) with reverse transcriptase and oligo-dT (High Capacity cDNA reverse transcription kit, Applied Biosystems). RT-qPCR was performed in optical 96-well plates using SYBR® green in a Light Cycler 480 (Roche). Primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The *β-tubulin2* gene (At5g05620) was used to normalize the transcript level in each sample. Primers used for RT-qPCR and stem-loop RT-qPCR are listed in **Supplementary table S2**. Accumulation of mature miR827 was determined by stem-loop reverse transcription quantitative PCR (Varkonyi-Gasic *et al.*, 2007). At least 3 biological replicates were analyzed per genotype and condition, each replicate consisting of leaves

from at least 3 independent plants. Two-way analysis of variance (ANOVA) was used to analyze data.

Hormone determination

The rosettes of three-week-old WT (Col-0) and *nla* plants were analyzed by LC-MS for SA, SAG, JA, OPDA and camalexin content as previously described in Sánchez-Bel *et al.*, 2018. Briefly, 30 mg of freeze dried material was extracted with MeOH:H2O (30:70) containing 0.01% of HCOOH containing a mixture of 10 ug. L-1 of the internal standards salicylic acid-d 5 (SA-d5) and dehydrojasmonic acid (Sigma-Aldrich). Following extraction, samples were centrifuged (15.000 rpm, 15 min) and filtered through regenerated cellulose filters. An aliquot of 20 ul was injected into a UPLC (Waters Aquity) interfaced with a Xevo TQ-S Mass Spectrometer (TQS, Waters). Hormones were quantified by contrasting with an external calibrarion curve of pure chemical standards of SA, SAG, JA, OPDA and camalexin. Sample separation was performed with a LC Kinetex C18 analytical column of a 5 µm particle size, 2.1 100 mm (Phenomenex). Cinematographic and TQS conditions were performed as described in Sanchez-Bel *et al.* (2018). At least 6 biological replicates were analyzed per genotype and condition, each replicate consisting of leaves from at least 3 independent plants. The plant material was lyophilized prior analysis. Two-way analysis of variance (ANOVA) followed by HSD (Honestly-Significant-Difference) Tukey's test was used to analyze data.

RESULTS

NLA and *MIR827* expression is regulated during fungal infection.

Arabidopsis thaliana contains a single copy of *MIR827* gene, whose expression is induced in Pi limiting conditions, resulting in down-regulation of *NLA* expression (Hsieh *et al.*, 2009). In this work, we investigated whether pathogen infection has an effect on *MIR827* and *NLA* expression in Arabidopsis. For this, Arabidopsis plants were challenged with the fungal pathogen *Plectosphaerella cucumerina.* The Arabidopsis/*P. cucumerina* pathosystem is a well-established model for studies on basal resistance to infection by necrotrophic fungi (Sanchez-Vallet *et al.*, 2010). The accumulation of miR827 precursor and mature transcripts significantly increased in *P. cucumerina*-infected plants compared with non-infected plants, whereas *NLA* was repressed in the fungal-infected plants (**Figure 1A**).

The effect of treatment with fungal elicitors was also examined. Perception of pathogen-derived molecules (PAMPs, for Pathogen-Associated Molecular Patterns, also named as elicitors) is known to trigger the induction of general plant defense responses, or PTI. As shown in **Figure 1B,** treatment with a crude preparation of *P. cucumerina* elicitors was accompanied by up-regulation of *MIR827* expression and down-regulation of *NLA* expression.

From these results it is concluded that MIR827 and NLA expression is regulated during pathogen infection. Since *NLA* is known to be the target gene of miR827, induction of *MIR827* expression during *P. cucumerina* infection or elicitor treatment might account, at least in part, for the reduced expression of *NLA*.

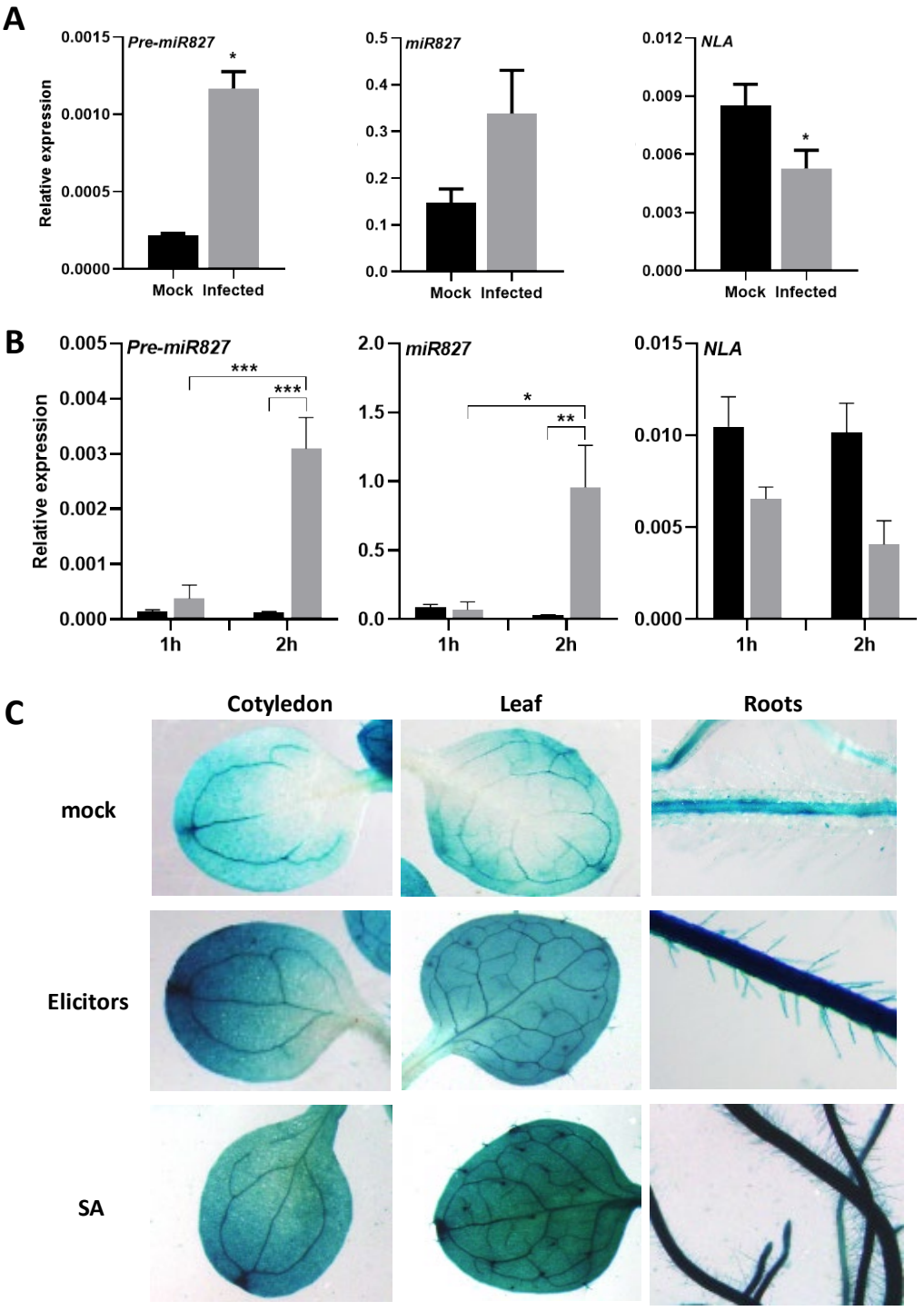


Figure 1. *MIR827* and *NLA* expression in response to infection with *P. cucumerina* and treatment with *P. cucumerina* elicitors. Plants were grown *in vitro* in modified Hoagland half strength under low Pi conditions ($P_{0.05}$ mM) for 7 days.

A and **B**. Accumulation of pre-miR827, mature miR827 and *NLA* transcripts in response to inoculation with *P. cucumerina* spores ($5 \cdot 10^5$ spores/ml) at 48hpi **A**. or treatment with elicitors obtained from this fungus (300 µg/ml elicitors) at the indicated times of treatment **B**. The level of transcripts was determined by RT-qPCR (pre-miR827) and stem-loop RT-qPCR (miR827). Black bars, mock-inoculated plants. Grey bars, *P. cucumerina*-inoculated or elicitor-treated plants. Histograms show the mean ± SEM. Statistical significance was determined by ANOVA (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$).

C. Histochemical analysis of GUS activity in tissues of *miR827prom::GUS* plants that have been grown under low supply (0.05mM). Plants were mock-inoculated or treated with *P. cucumerina* elicitors (300 µg/ml elicitors; 60 min of treatment), or SA (0.1 mM SA; 12 h of treatment). Representative images are shown. Two independent lines were assayed which gave similar results. The analysis of GUS activity in *miR827prom::GUS* plants that have been grown under P0.25 supply is presented in **Supplemental Figure S2.**

To get further support of elicitor-responsiveness of *MIR827*, we generated transgenic lines expressing the *GUS* reporter gene under the control of the *MIR827* promoter (*MIR827prom::GUS* lines). As control, transgenic Arabidopsis plants expressing the *GUS* reporter gene under the control of the *35S Cauliflower Mosaic Virus 35S* promoter (*35Sprom::GUS* plants) were also examined. Arabidopsis plants were grown under two different Pi regimes, namely 0.05 mM and 0.25 mM Pi (low and sufficient conditions; hereafter $P_{0.05}$ and $P_{0.25}$ plants). Pi content determination confirmed reduced Pi level in leaves of $P_{0.05}$ plants compared with $P_{0.25}$ plants (**Figure S1A**). As expected, the accumulation of precursor and mature miR827 transcripts substantially decreased when increasing Pi supply, while *NLA* expression increased (**Figure S1B**).

When growing *miR827prom::GUS* lines plants under low Pi supply (0.05 mM Pi), GUS activity was observed in cotyledons, leaves and roots (**Figure 1C,** mock). In *MIR827prom::GUS* plants grown under high Pi supply (2 mM Pi), however, GUS activity was detected only at the distal part of cotyledons and leaves, but not in roots (**Figure S2A**). To note, treatment with fungal elicitors caused a substantial increase in the activity of the *MIR827* promoter in all Arabidopsis tissues (**Figure 1C**), which is in agreement with results obtained by expression analysis (**Figure 1B**). *35Sprom::GUS* plants served as controls (**Figure S2B, C**).

The elicitor-responsiveness that occurs in *MIR827prom::GUS* plants is also consistent with the presence of sequence motifs (*cis*-elements) that are known to be involved in pathogen-regulated genes in the *MIR827* promoter (**Figure S3; Table S1**). In particular, multiple W box TGAC core elements that mediate the transcriptional activation of pathogen- and elicitor-regulated genes, as well as SA-responsive *cis*-elements, were identified in the *MIR827* promoter region (e.g. WBOXATNPR1, TTGAC; ELRECOREPCRP1, TTGACC) (**Figure S3; Table S1**). SA-responsiveness of the *MIR827* promoter was confirmed in SA-treated *MIR827prom::GUS* plants (**Figure 1C**). As expected, the P1BS

element present in phosphate starvation responsive genes was also identified in the *MIR827* promoter (**Figure S3; Table S1**). Together, these findings suggest that changes in transcript abundance of *MIR827* in response to treatment with fungal elicitors or SA treatment are largely controlled at the transcriptional level.

Resistance to infection by fungal pathogens in *nla* and miR827 overexpressor plants

To investigate whether *NLA*, and its regulatory miRNA, miR827, play a role in Arabidopsis immunity we examined disease resistance in *nla* mutant and miR827 overexpressor plants (miR827 OE). As miR827 down-regulates *NLA* expression, transgenic lines overexpressing miR827 are expected to exhibit a disease phenotype similar to that of the loss-of-function mutation of *nla*. The *nla* mutant used for these studies, *nla-1* (Col-0 background) is a RING-domain deletion mutant caused by deletion of the third and fourth exon which was described elsewhere (Peng *et al.*, 2007; Kant *et al.*, 2011; Lin *et al.*, 2013) (**Figure S4A**). As previously reported, the *nla* plants accumulated Pi in their leaves, and showed down-regulation of *NLA* expression compared with wild-type plants (Lin *et al.*, 2013) (**Figure S4B**). The production of miR827 OE plants was previously described, Kant *et al*., 2011. As expected, miR827 OE plants accumulated pre-miR827 transcripts and had reduced *NLA* expression, these plants also accumulating Pi in their leaves (**Figure S4C**). Compared with wild-type plants, no visible morphological changes were observed in *nla* and miR827 OE plants at the time of inoculation with fungal spores (3 week-old plants) (**Figure S4D**). Interestingly, the *nla* plants consistently showed enhanced resistance to infection by *P. cucumerina* compared with the wild-type plants (**Figure 2A**, left and middle panels). Enhanced resistance to infection by *P. cucumerina* was also observed in miR827 OE plants (**Figure 2A,** right panel). Quantification of fungal biomass and determination of plant survival confirmed resistance to *P. cucumerina* in both *nla* and miR827 OE plants (**Figure 2B**). Moreover, trypan blue staining of infected leaves revealed extensive fungal growth in leaves of wild-type plants, but not in leaves of *nla* plants (**Figure 2C**). Here it should be mentioned that Hewezi *et al.*, 2016 reported that Arabidopsis plants overexpressing miR827 displayed reduced plant size. Under our

experimental conditions, however, no phenotypic differences between wild-type and miR827 OE plants were observed at the time of inoculation with *P. cucumerina* spores.

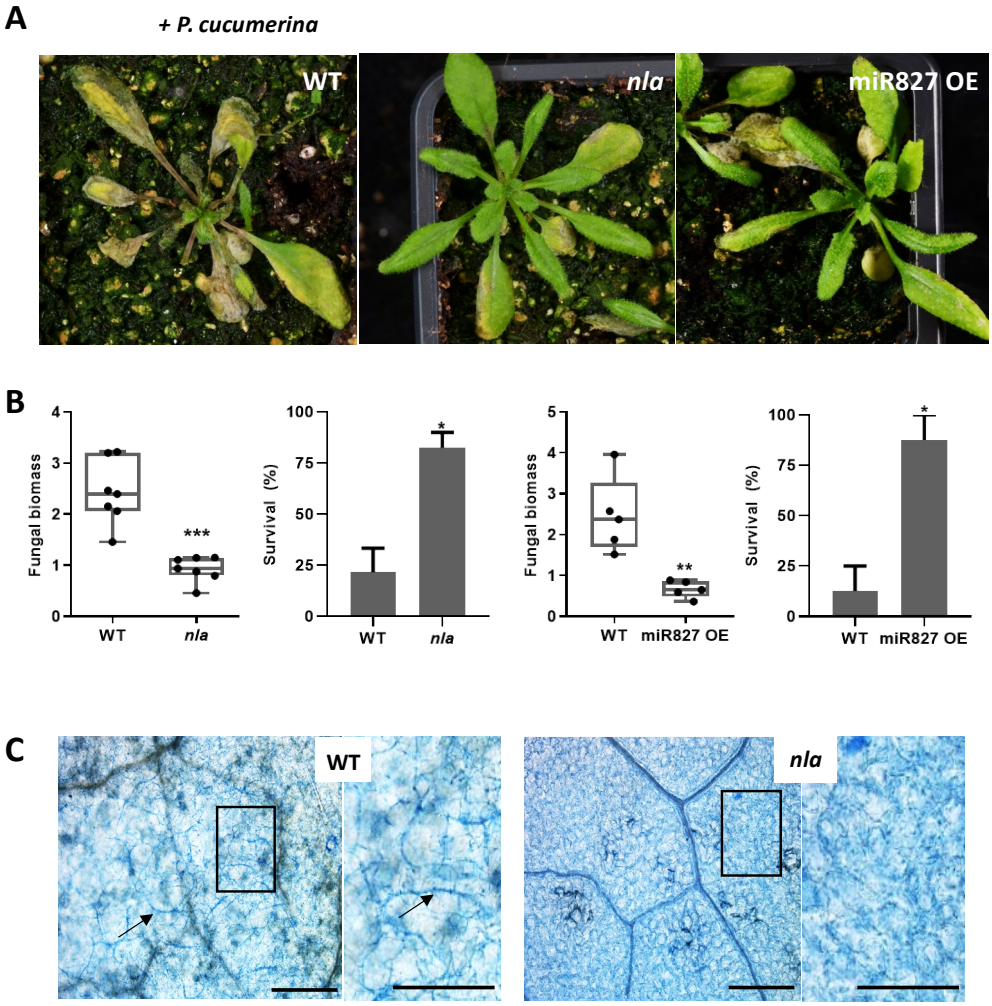


Figure 2. Resistance of *nla* and miR827 OE plants to infection by necrotrophic fungal pathogen *P. cucumerina*. Three-week-old plants were mock-inoculated or inoculated with *P. cucumerina* ($5 \cdot 10^5$ spores/ml). Three independent infection experiments were carried out with similar results (at least 24 plants per genotype each experiment).

A. Phenotype of *P. cucumerina*-infected wild-type, *nla* and miR827 OE plants. Images were taken at 7 days after inoculation (dpi).

B. Fungal biomass and survival of *nla* and miR827 OE plants. Quantification of fungal DNA was performed by qPCR using specific primers of *P. cucumerina* (Soto-Suárez *et al.*, 2017) at 7 dpi. Survival was determined at 7 dpi.

C. Trypan blue staining of leaves from *P. cucumerina*-infected *nla* plants at 7 dpi. Arrows indicate fungal hyphae. Scale bars represent 200 µm. Higher magnifications are shown (right panels; bars, 100 µm).

Furthermore, *nla* plants exhibited resistance to infection by the hemibiotrophic fungus *Colletotrichum higginsianum*, which was confirmed by the amount of fungal biomass and percentage of diseased leaves (**Figure 3)**. *C. higginsianum* causes the anthracnose leaf spot disease of *Brassica* species, including *A. thaliana* (O'Connell *et al.*, 2004). Subsequent studies in this work were conducted on *nla* mutant plants.

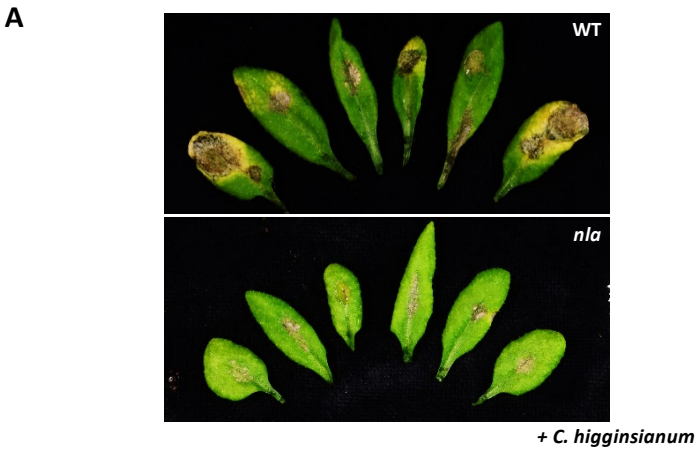


Figure 3. Resistance of *nla* plants to infection by the hemibiotrophic pathogen *C. higginsianum.* Three independent infection experiments were carried out with similar results (at least 24 plants per genotype each experiment).

A. Phenotype of wild-type and *nla* plants that had been inoculated with *C. higginsianum* spores (4 x 10^6 spores/ml), or mock inoculated. Pictures were taken at 7 days after inoculation.

B. Fungal biomass and lesion area was determined at 7 dpi. Quantification of fungal DNA was performed by qPCR using specific primers of *C. higginsianum* (Soto-Suárez *et al.*, 2017).

Callose and ROS accumulation in *nla* plants in response to *P. cucumerina* infection

It is well known that callose and ROS accumulation are important factors in the Arabidopsis defense response to infection by *P. cucumerina* (Luna *et al.*, 2011; Wang *et*

al., 2021). ROS function as positive signals for callose accumulation, as well as for the induction of defense responses in response to pathogen infection or treatment with elicitors (Zhang *et al.*, 2007; Daudi *et al.*, 2012).

In this work, aniline blue was used to visualize callose accumulation in leaves of wild-type and *nla* plants that have been inoculated with *P. cucumerina* spores, or mock-inoculated. Neither wild-type nor *nla* plants showed callose deposition in the absence of pathogen infection (mock conditions) (**Figure 4A**). Notably, *nla* plants infected with *P. cucumerina* showed significantly increased callose deposits compared with *P. cucumerina*-infected wild-type plants (**Figure 4A**). Callose deposition was quantified by determining the relative number of fluorescent pixels on digital photographs that corresponded to pathogen-induced callose. This analysis showed that callose accumulation was significantly higher in *nla* plants than in the wild-type plants (**Figure 4A,** right panel), which might well contribute to arrest pathogen growth in *nla* plants. The observation that callose does not accumulates in *nla* plants in the absence of pathogen infection suggest that this immune response is not constitutively active in *nla* plants.

Knowing that pathogen infection (or elicitor treatment) reduces *NLA* expression, and that *nla* plants accumulating Pi exhibited pathogen resistance, these plants also showing greater callose accumulation during pathogen infection, it was of interest to determine whether callose accumulation is affected by Pi supply. Towards this end, wild-type and *nla* plants were grown *in vitro* under different Pi concentrations (0.05 mM, 0.25mM and 2 mM Pi). Measurement of Pi content confirmed that increasing Pi supply to plants results in higher leaf Pi content (e.g. plants grown in 0.25 and 2 mM Pi) (**Figure S5**). In the absence of pathogen infection, both wild-type and *nla* plants exhibited small fluorescent callose spots at the highest Pi condition (2 mM Pi), but not at lower Pi concentrations (0.05 and 0.25 Pi) (**Figure 4B**). Upon pathogen challenge, more ring-shaped callose deposits were clearly visible in *P. cucumerina*-infected WT and *nla* plants which were more evident when Pi supply increased (**Figure 4B**). Quantification of callose deposition confirmed higher accumulation in *nla* plants compared with WT plants in response to infection (0.25 and 2 mM Pi conditions) (**Figure 4C**).

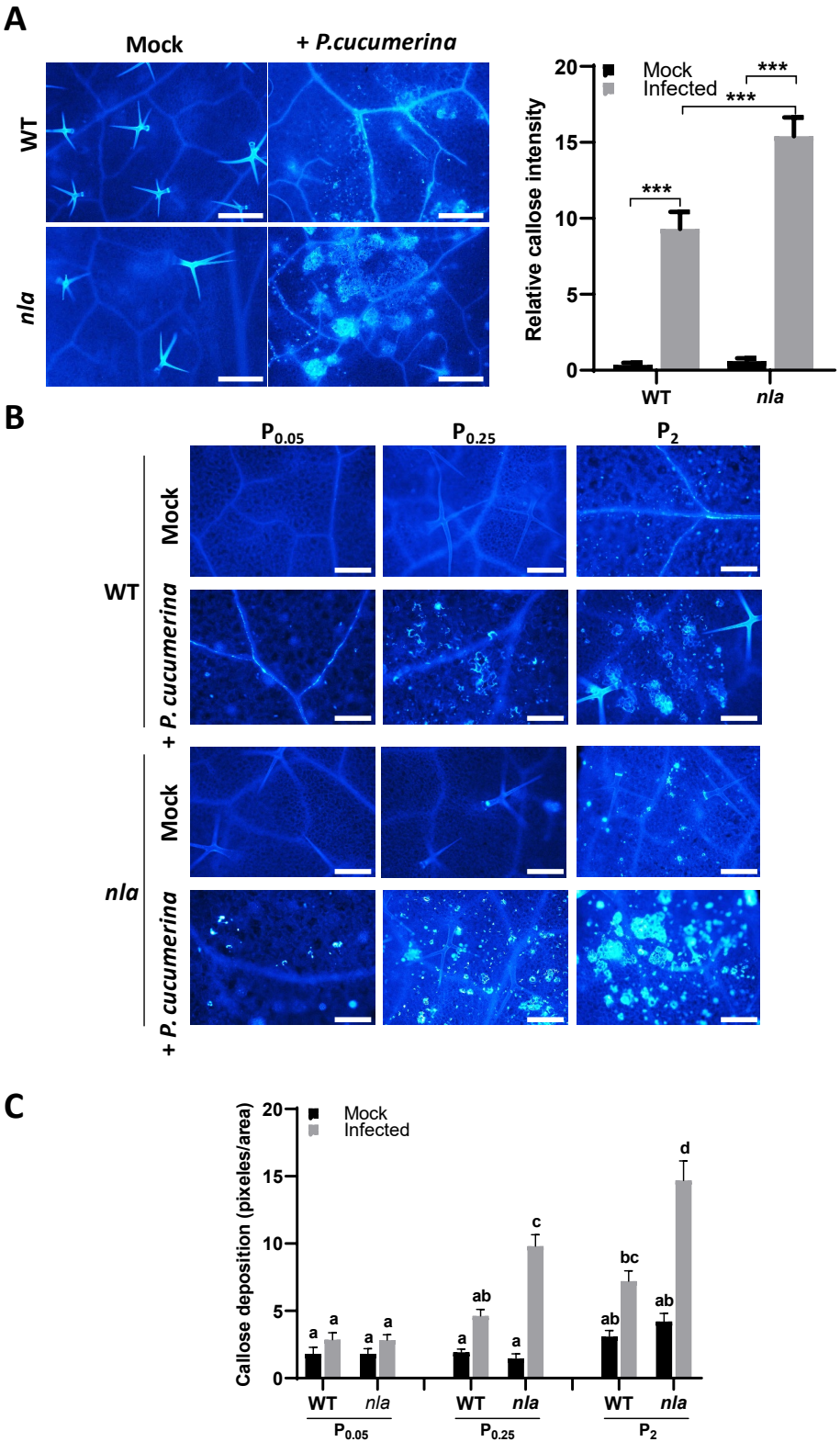


Figure 4. Callose deposition in *P. cucumerina*-infected leaves of wild-type and *nla* plants. Wild-type and *nla* plants were inoculated with *P. cucumerina* spores (5 x 10^5 spores/ml), or mock-inoculated. Aniline blue staining was carried out at 48 hpi. Representative results from one of three independent infection experiments that gave similar results are shown (at least 12 plants each experiment).

A. Micrographs of aniline blue stained leaves. (left panel). Bars represent 200 µm. Right panel, pathogen-induced callose deposition was quantified by determining the relative number of fluorescent pixels on digital micrographs of infected leaves (right panel). Bars represent mean ± SEM.
B. Callose accumulation in Arabidopsis plants that have been grown *in vitro* under increasing Pi supply for 7 days (0.05 mM, 0.25 mM and 2 mM Pi), and then inoculated with *P. cucumerina* spores, or mock-inoculated.
C. Quantification of callose deposition in wild-type and *nla* plants (same conditions as in B).

Not only callose deposition, but also ROS production was found to be higher in *nla* plants than in wild-type plants, as revealed by H_2DCFDA staining (**Figure 5**). This increase was more evident in plants grown under high Pi supply (P_2 plants). That increasing Pi supply is accompanied by an increase in ROS accumulation in wild-type plants was also found in studies presented in Chapter 1 in the present PhD Thesis.

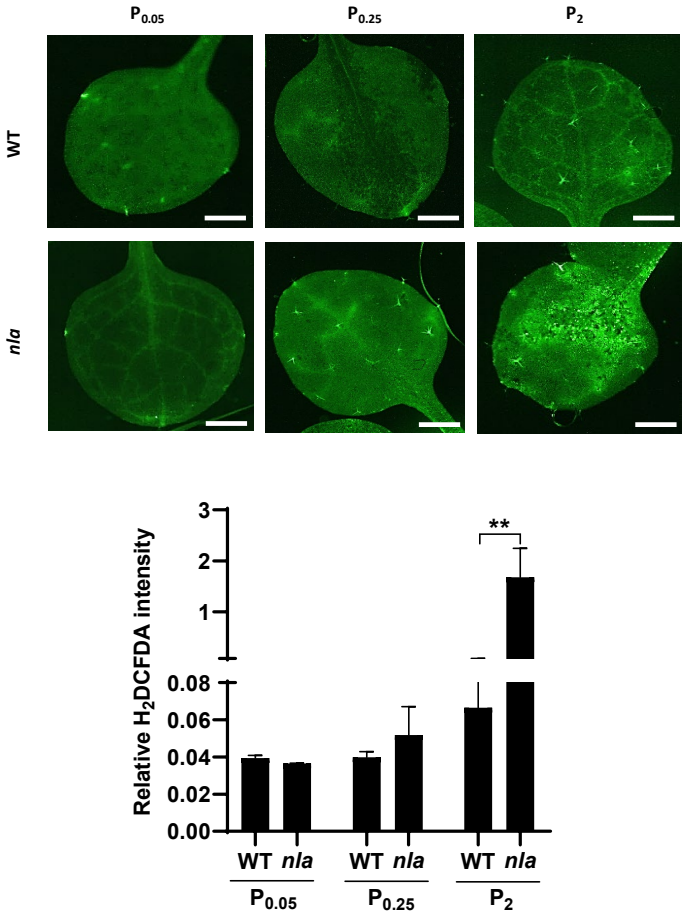


Figure 5. Enhanced accumulation of ROS in wild-type and *nla* plants that have been grown *in vitro* under increasing Pi supply (0.05 mM, 0,25 mM, 2 mM Pi). Visualization of H_2O_2 accumulation was carried out using the fluorescent probe H_2DCFDA. Scale bars represent 1 mm. H_2DCFDA was quantified by determining the relative number of fluorescent pixels on digital micrographs of infected leaves (right panel).

Collectively, these results demonstrated higher callose deposition and ROS production in *nla* plants compared with wild-type plants, and that callose and ROS accumulation notably increased in *nla* plants during pathogen infection.

The Arabidopsis mutant *nla* accumulates SA and JA

To further understand the mechanisms involved in resistance to pathogen infection in *nla* plants, we investigated the expression of SA- and JA-regulated defense genes in these plants. In the absence of pathogen infection, the SA markers *PR1* and *NPR1* showed a higher basal expression in *nla* plants, their expression being further induced upon *P. cucumerina* infection in both wild-type and *nla* plants (**Figure 6A**). Similar levels of pathogen-inducible *PR1* and *NPR1* expression were observed in wild-type and *nla* plants (**Figure 6A**).

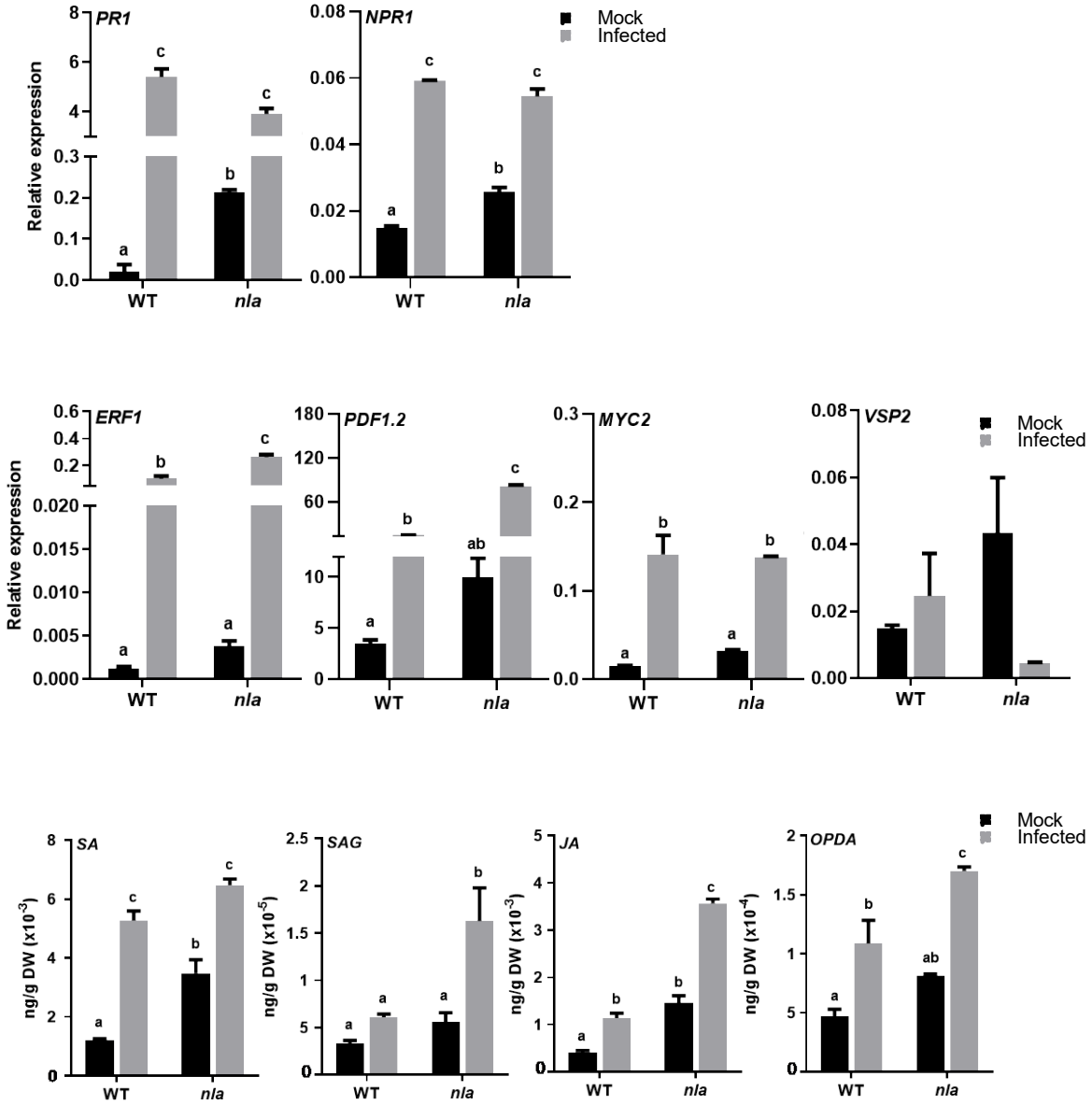


Figure 6. Expression of defense marker genes of the SA and JA signalling pathways and accumulation of SA and JA in wild-type and *nla* plants. Transcript levels in A and B were determined by RT-qPCR in mock-inoculated and *P. cucumerina*-inoculated plants at 48hpi (black and grey bars, respectively). Bars represent mean ± *SEM* (three biological replicates and three plants per replicate). Letters indicate statistically significant differences (ANOVA, HSD Tukey's test, $P \leq 0.05$).

A. Genes in the SA signaling pathway (*PR1*, *NPR1*)

B. Genes in the ERF1/PDF1.2 and MYC2/VSP2 branch in the JA signaling pathway.

C. Levels of SA, SAG, JA and OPDA.

We then examined expression of genes in the two branches of the JA signaling pathway, the MYC2 branch and ERF branch, which are regulated by AtMYC2 (basic helix-loop-

helix-leucine zipper transcription factor) and AtERF1 (belonging to the APETALA/ERF transcription factor family), respectively (Lorenzo *et al.*, 2004). The marker gene commonly used for the ERF branch is *PDF1.2* (*Plant Defensin 1.2*), whereas the induction of *VSP2* (*Vegetative Storage Protein 2*) serves as marker of the MYC branch (Lorenzo *et al.*, 2004; Pieterse *et al.*, 2012; Wasternack and Hause, 2013; Zhang *et al.*, 2017). *ERF1* and *PDF1.2* expression slightly increased in *nla* plants compared with wild-type plants (non-infected in both cases) (**Figure 6B**). In *nla* plants, a tendency for a higher expression of *MYC2* and *VSP2* compared with wild-type plants was also observed (in the absence of pathogen infection). *P. cucumerina* infection further induced *ERF1*, *PDF1.2* and *MYC2* expression in both genotypes (**Figure 6B**). In contrast, an opposite trend was observed in *VSP2* in the response of wild-type and *nla* plants to pathogen infection (down-regulation and up-regulation, respectively) (**Figure 6B**). Similar results were observed in miR399 OE and *pho2* Arabidopsis plants (both accumulating higher Pi than wild-type plants, as the *nla* mutant) in which the ERF1/PDF1.2 branch positively responded to *P. cucumerina* infection, while the MYC2/VSP2 branch was negatively regulated during infection (see Chapter 1).

The observation that *nla* plants showed greater expression of SA- and JA-regulated defense genes in the absence of pathogen infection, raised the possibility that SA and JA levels would be higher in *nla* plants compared with wild-type plants. Accordingly, we measured levels of SA (and the SA glucoside SAG) and JA (and its biosynthetic precursor OPDA), in wild-type and *nla* plants that have been inoculated with *P. cucumerina* or mock-inoculated. Our results revealed that, in the absence of pathogen infection, *nla* plants accumulated higher levels of SA and JA compared to wild-type plants (**Figure 6C**), which is consistent with the expression pattern of SA- and JA-regulated defense genes in these plants. In response to pathogen infection, wild-type and *nla* plants displayed similar levels of SA, but the JA level was higher in *nla* plants than in wild type plants (**Figure 6C**). As for SAG, the storage form of SA, a higher accumulation in *P. cucumerina*-infected *nla* plants points to a tight control of SA content in these plants (**Figure 6C**). Thus, compared with wild-type plants, *P. cucumerina* infection is accompanied by a higher accumulation of JA in *nla* plants compared with wild-type plants, whereas SA level was similar in both genotypes (under infection in both cases). As SA and JA content is

already high in *nla* plants in the absence of infection, it is tempting to hypothesize that *nla* plants are able to respond in a more rapid and efficient manner once the pathogen is recognized. A more detailed study on the induction kinetics of defense-related genes is required to determine whether they are induced earlier in *nla* than in wild-type plants in response to pathogen infection.

Accumulation of camalexin in *nla* mutant plants

Camalexin is a phytohormone that plays an important role in the defense response of Arabidopsis plants against necrotrophic fungi, such as *B. cinerea* or *P. cucumerina* (Barrlen *et al.*, 2007; Sanchez-Vallet *et al.*, 2010). On the other hand, it has long been recognized that local resistance to *B. cinerea* depends on JA and SA signaling pathway as well as on the synthesis of the phytoalexin camalexin (Ferrari *et al.*, 2003). A modulation of camalexin formation by components of the JA signaling pathway was also described (Rowe *et al.*, 2010; De Geyter *et al.*, 2012). Accordingly, in this study, we examined the expression of genes involved in camalexin biosynthesis in *nla* mutant plants, these plants accumulating higher levels of SA and JA. Genes encoding cytochrome P450 enzymes involved in the conversion of tryptophan to camalexin, namely *CYP79B2*, *CYP79B3*, *CYP71A13* and *CYP71B15/PAD3* are indicated in (**Figure 7A**). Their expression was determined in *nla* and wild-type plants that have been inoculated with *P. cucumerina* spores, or mock inoculated**.** As it is shown in **Figure 7B**, all camalexin biosynthesis genes reached higher levels of expression in the fungal-infected *nla* plants compared with the fungal-infected wild-type plants. Consistent with the observed super-induction of camalexin biosynthesis genes in the *P. cucumerina*-infected *nla* plants, these plants had a higher content of camalexin than wild-type plants (**Figure 7C**). A higher accumulation of camalexin in *nla* plants might well contribute to the resistance phenotype that is observed in these plants.

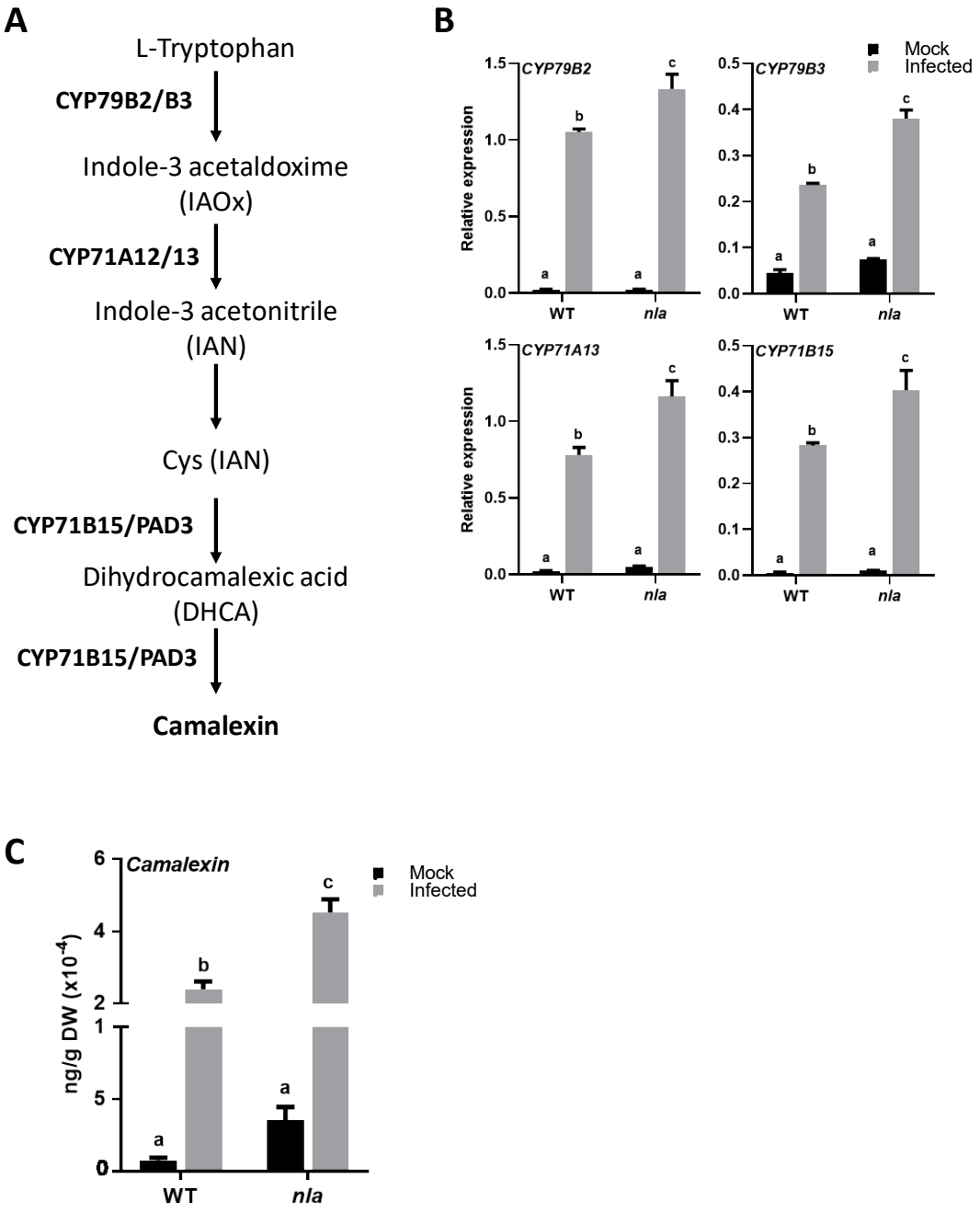


Figure 7. Expression of camalexin biosynthesis genes (A) and camalexin accumulation (B) in mock-inoculated and *P. cucumerina*-inoculated wild-type and *nla* plants. Six biological replicates (three plants per replicate were examined). Bars represent mean ± *SEM*. Letters indicate statistically significant differences (ANOVA, HSD Tukey's test, $P \leq 0.05$).

A. Camalexin biosynthesis scheme.

B. Transcript levels of *CYP79B2*, *CYP79B3*, *CYP71A13* and *CYP71B15* were determined by RT-qPCR in mock-inoculated and *P. cucumerina*-inoculated plants.

C. Accumulation of camalexin.

DISCUSSION

Plants are subjected to a plethora of environmental cues to which they must respond in an efficient and coordinated manner. Upon pathogen infection, plants are capable of responding to pathogen attack through the activation of a large array of defense mechanisms that are triggered once the plant senses the invader. However, resistance to pathogens can be attenuated or strengthened with co-occurrence of additional stress factors, including nutrient stress. Although evidence support that nutrient deficiency or excess might influence the expression of plant immune responses, how pathogen-induced signaling pathways converge with signaling pathways triggered by other types of stresses still remain obscure.

In this work, we report that the miR827/NLA regulatory module plays a role in Arabidopsis immunity, thus, reinforcing the notion that cross-talk between Pi signaling pathways and immune responses exists in plants. So far, miR827 and NLA function has been typically associated to PSR, NLA being also involved in adaptation to nitrate limitation in Arabidopsis plants (Peng *et al.*, 2007). We show that an increase in Pi content caused either by miR827 overexpression or loss-of-function of *NLA* positively regulates defense responses and confers resistance to infection by pathogenic fungi with a necrotrophic (*P. cucumerina*) or a hemibiotrophic (*C. higginsianum*) lifestyle. Both fungal infection and treatment with fungal elicitors results in up-regulation of *MIR827* expression and down-regulation of NLA, the miR827 target gene. Histochemical analysis of *miR827prom::GUS* plants revealed that *MIR827* is transcriptionally activated not only by treatment with fungal elicitors but also by treatment with SA which is consistent with the presence of elicitor- and SA-responsive *cis* elements in the *MIR827* promoter.

Several lines of evidence support that the NLA contributes to disease resistance in Arabidopsis by negatively regulating immune responses. First, loss-of-function of *NLA,* as well as miR827 overexpression, confers resistance to infection. Second, resistance to pathogen infection in *nla* plants is associated with a stronger accumulation of callose and ROS. In this respect, the relevance of ROS production and callose deposition in resistance to pathogen infection in Arabidopsis, including *P. cucumerina* infection, is well established (Luna *et al.*, 2011; Pastor-Fernández *et al.*, 2019; Lee *et al.*, 2020). Similarly,

Pi treatment in Arabidopsis plants was found to foster ROS and callose accumulation and resistance to infection by the fungal pathogens *(P. cucumerina, C. higginsianum)* (see Chapter 1). Third, *nla* plants showed an increase in SA and JA content under non-infection (SA, JA) or infection conditions (JA). These findings correlated well with a higher expression of marker genes of SA- and JA-dependent signaling pathways. An opposite regulation of genes in the two branches of the JA signaling pathway occurs in *nla* and wild-type plants in response to *P. cucumerina* infection. Whereas pathogen infection induces *PDF1.2* (marker of the ERF1 branch), *VSP2* (maker of the MYC2 branch) is down regulated by infection in *nla* plants. An opposite response in the expression of genes in the two branches of the JA-signaling pathway was also observed in *pho2* mutant plants upon infection with *P. cucumerina*, the *pho2* plants also accumulating Pi in leaves (Chapter 1).

As a further confirmation of the implication of *NLA* in the control of defense responses, the *nla* plants accumulated higher levels of camalexin in their leaves (non-infection and infection conditions) which could be also inferred from the observed superactivation of camalexin biosynthesis genes in these plants. Camalexin plays a key role in Arabidopsis defense against *P. cucumerina*, and the *in vitro* antifungal activity of camalexin against *P. cucumerina* has been demonstrated (Sanchez-Vallet *et al.*, 2010). Also, *cyp79B2 cyp79B3* mutants were found to be more susceptible to *P. cucumerina* infection than wild-type plants (Sanchez-Vallet *et al.*, 2010). Thus, superinduction of camalexin biosynthesis genes, in particular *CYP79B2* and *CYP79B3*, and subsequent accumulation of camalexin, might explain the phenotype of disease resistance observed in *nla* mutant plants. Altogether, results here presented support that *nla* plants exhibit stronger immune responses. Enhanced resistance to pathogen infection in *nla* plants might be attributable to high basal expression and/or stronger induction of immune responses and accumulation of the defense-related hormones SA and JA. In other studies, Yaeno and Iba, 2008 reported that the *benzoic acid hypersensitive1-Dominant* (*bah1-D*) mutant (an allele of *nla* containing a T-DNA fragment inserted into the 5'-untranslated region of *NLA*) accumulated higher levels of SA after application of benzoic acid and exhibited enhanced resistance to *P. syringae* pv *tomato* DC3000 (Yaeno and Iba, 2008). Although the exact mechanisms by which *NLA* regulates defense responses remain unknown,

results presented in this work on resistance to infection by fungal pathogens in *nla* mutant plants, together with those previously reported on resistance to bacterial infection, suggest that *NLA* functions as a negative regulator in Arabidopsis immunity. Contrary to this, inactivation of *NLA* expression was found to increase susceptibility to the cyst nematode *Heterodera schachtii* (Hewezi *et al.*, 2016). Together, results here presented reinforce the notion that Pi accumulation is an important factor in controlling Arabidopsis immune responses while further supporting the existence of links between phosphate homeostasis and disease resistance.

In Arabidopsis, Pi homeostasis is regulated via the integration of miRNA-mediated posttranscriptional mechanisms (miR399, miR827) as well as by ubiquitin-mediated posttranslational regulatory pathways (PHO2, NLA). Both PHO2 and NLA regulate the abundance of PHT1 transporters to maintain Pi homeostasis. It is then not surprising that features of the *nla* mutant resemble characteristics of the *pho2* mutant. Similar to the *nla* mutant, *pho2* plants accumulate Pi and exhibit resistance to infection by fungal pathogens (see Chapter 1). As in *nla* plants, suppression of *PHO2* expression increases SA and JA content, and causes superactivation of SA- and JA-dependent defense genes, with an opposite regulation in the two branches of the JA pathway during *P. cucumerina* infection (see Chapter 1). Thus, an increase in Pi content caused by mutations either in *PHO2* or *NLA* makes the plant to react to pathogen infection with stronger activation of defense responses and, hence, disease resistance. Further supporting regulation of immune responses by Pi, treatment with high Pi of Arabidopsis plants was also found to be accompanied by super-induction of defense genes and disease resistance (Chapter 1). As both *NLA* and *PHO2* mutation results in Pi accumulation, it is reasonable to assume that signaling pathways triggered by Pi accumulation interact with immune signaling pathways in Arabidopsis.

Regarding the mechanisms by which Pi accumulation might confer resistance to infection by fungal pathogens, several possibilities, not mutually exclusive, can be considered. On the plant side, increased supply of Pi might improve the physiological and metabolic status of the plant which would allow the plant to mount an effective immune response during pathogen infection. Another possibility is that Pi accumulation

might have a toxic effect on fungal growth. At present, however, it is not known whether Pi levels might affect *P. cucumerina* and *C. higginsianum* growth.

Thus, from what we have learned so far, nutrient supply and balanced plant nutrition plays a crucial role in disease resistance. How the nutritional status of a plant is going to affect disease resistance could be dependent on the plant species and the type of pathogen and/or pathogen lifestyle. Results here presented indicated that *nla* plants exhibit resistance to both necrotrophic and hemibiotrophic fungal pathogens, indicating that *nla*-mediated disease resistance, likely, is not dependent on the pathogen lifestyle. However, contrary to what is observed in Arabidopsis, Pi accumulation in rice leaves was found to increase susceptibility to infection by the rice blast fungus *M. oryzae* infection. This observation suggests that there is not a general model to predict the role of Pi in a given plant-pathogen interaction. Understanding how Pi signaling pathways interact with immune signaling pathways in different plant/pathogen interactions will help in designing strategies to improve disease resistance in plants.

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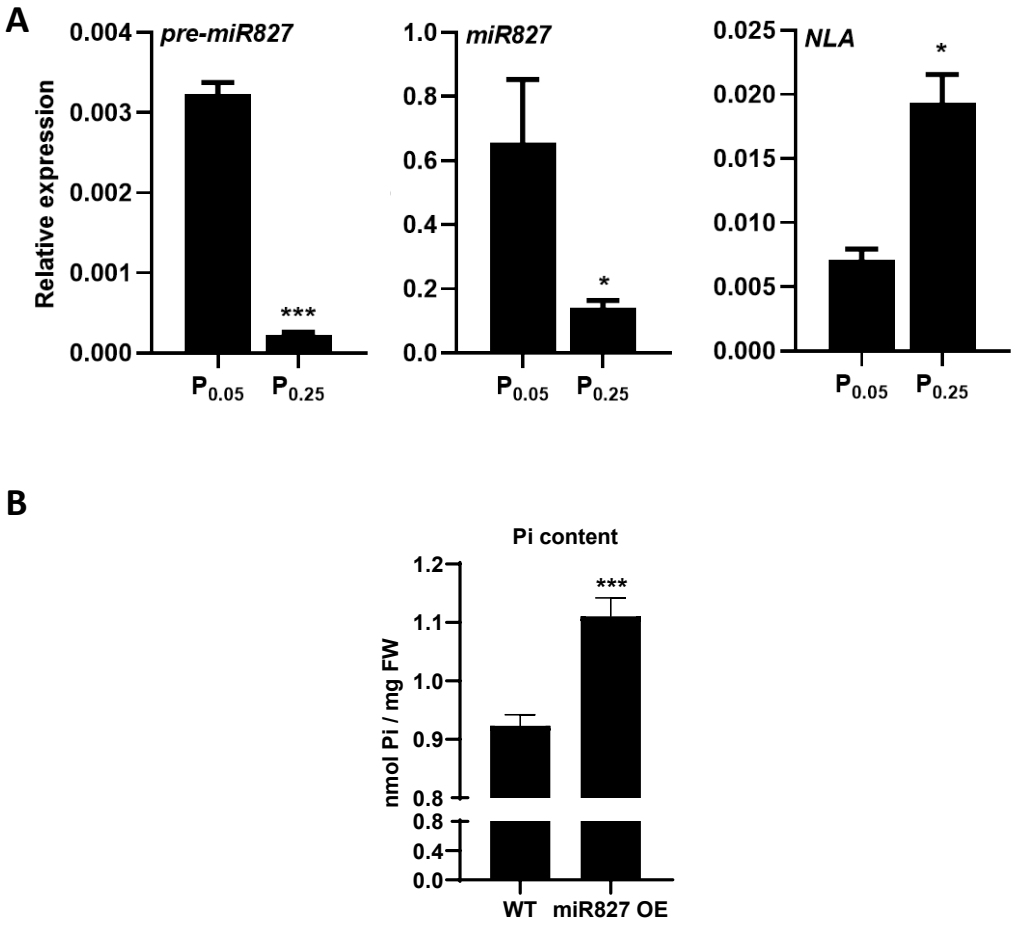
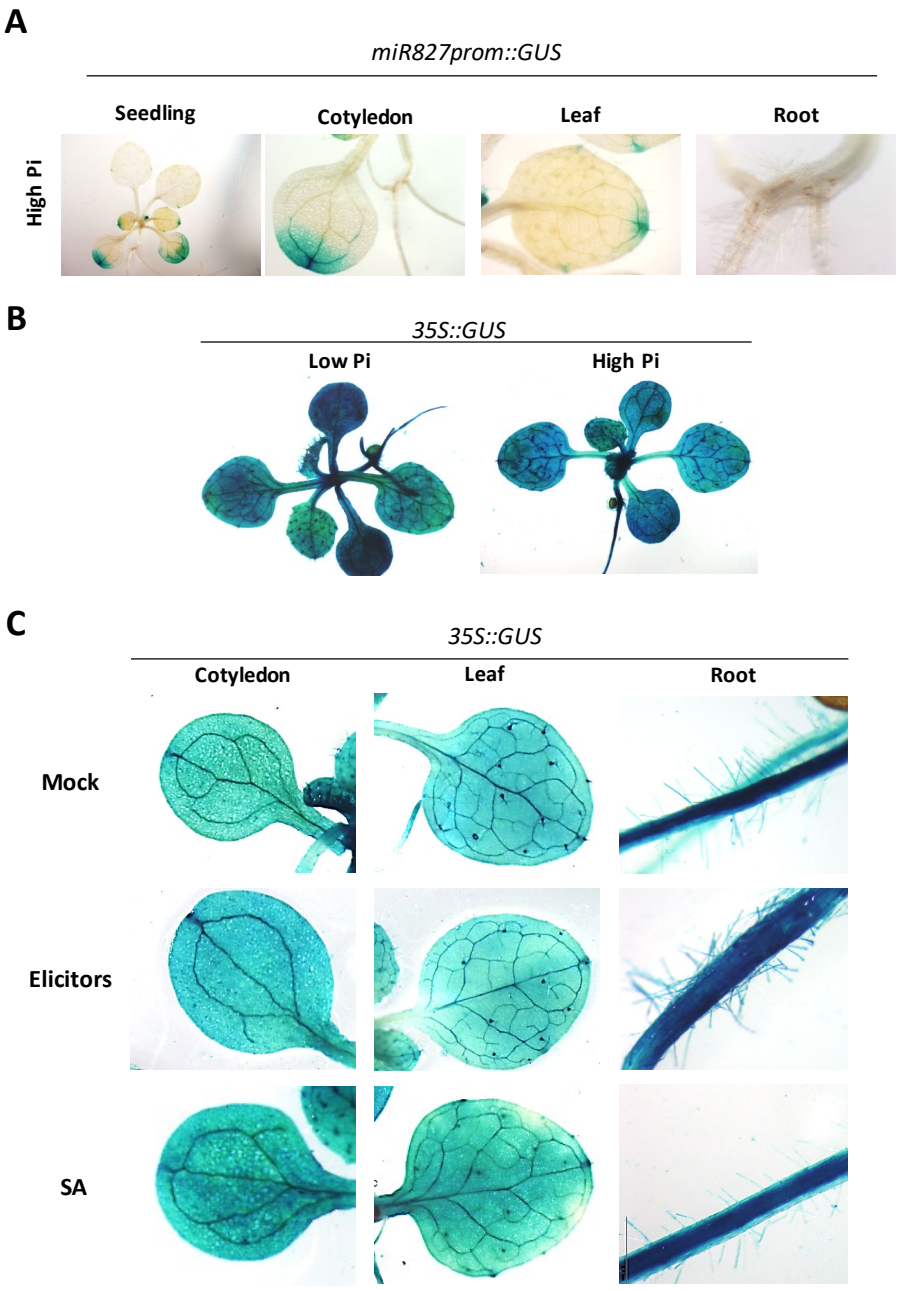
SUPPLEMENTAL MATERIAL



Supplemental Figure S1. *MIR827* and *NLA* expression in Arabidopsis plants that have been grown under low ($P_{0.05}$) or sufficient ($P_{0.25}$) Pi supply for 7 days. **A.** Accumulation of pre-miR827, mature miR827 and *NLA* transcripts. **B.** Pi content in $P_{0.05}$ and $P_{0.25}$ Arabidopsis plants. Bars represent mean ± *SEM* (n=3 and three plants per replicate) (*t* test, $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$).

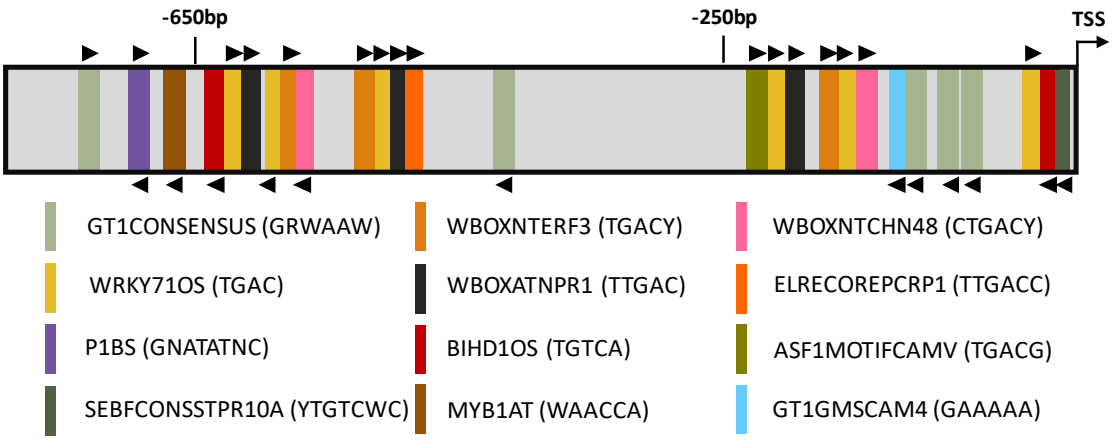


Supplemental Figure S2. Histochemical analysis of GUS activity of *miR827prom::GUS* and *35S::GUS* Arabidopsis plants.

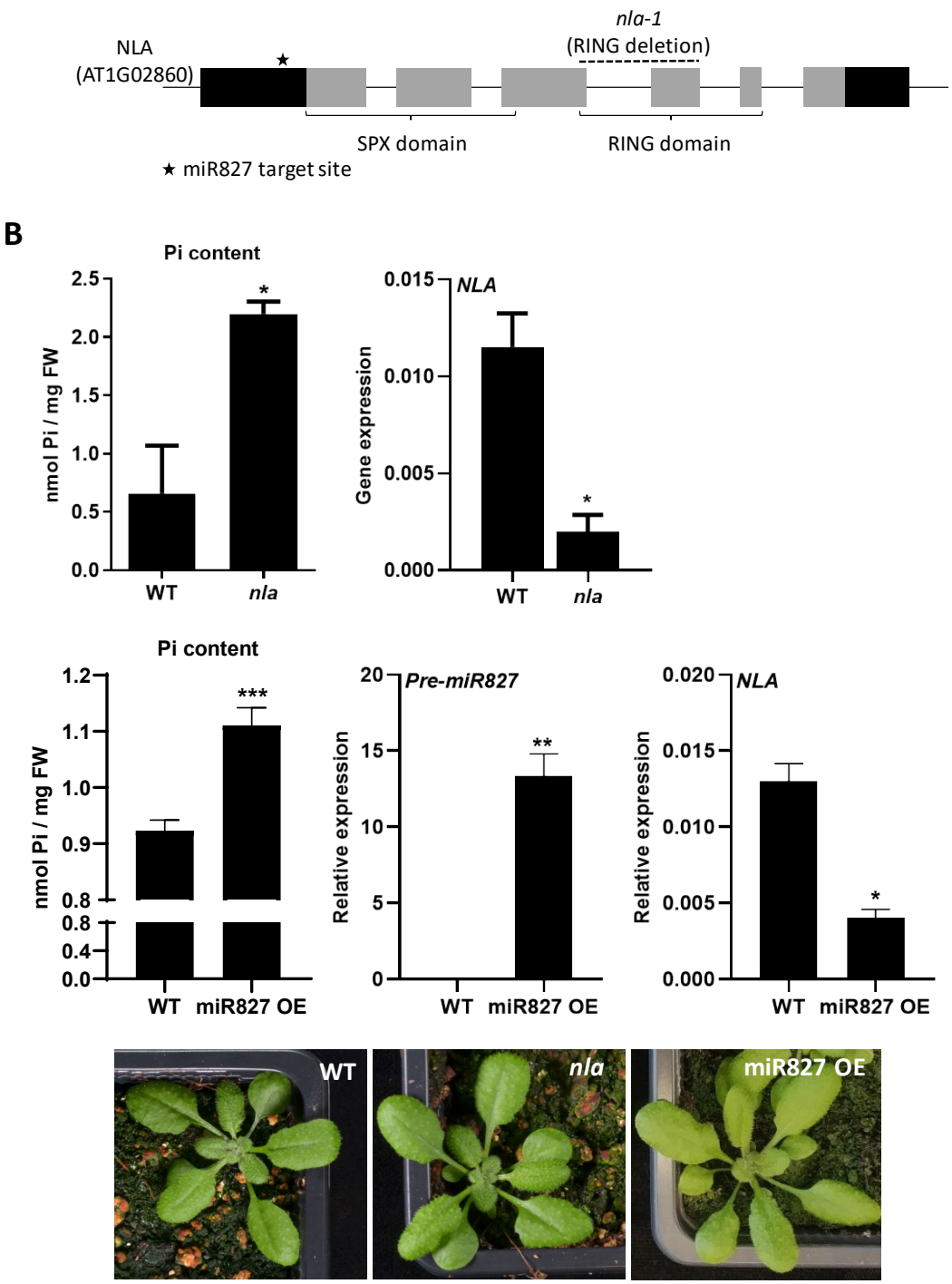
A. *miR827prom::GUS* plants grown under high Pi supply (2 mM Pi).

B. 35S::*GUS* Arabidopsis plants grown under low or high Pi supply (0.05 mM and 2 mM Pi, respectively)

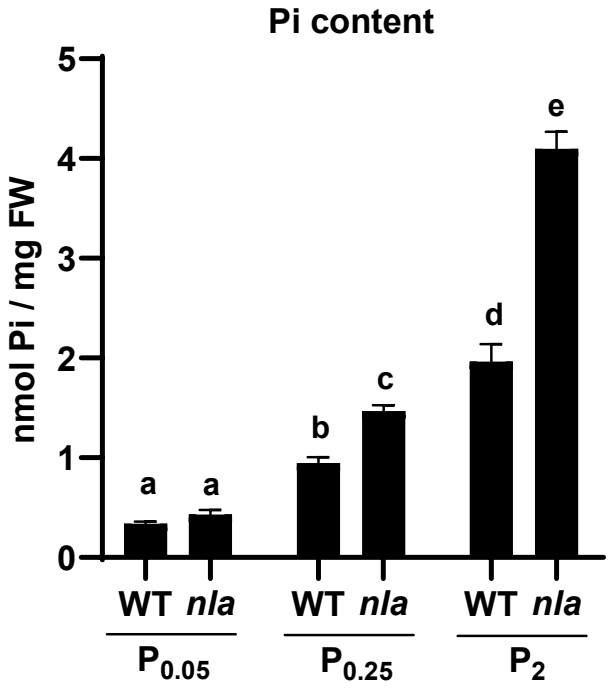
C. 35S::*GUS* plants grown under low Pi supply treated with elicitors prepared from the fungus *P. cucumerina* (0.3 mg/ml; 60 min of treatment) or SA (0.1 mM SA, 12h of treatment). Mock-inoculated plants served as a control.



Supplemental Figure S3. Structural features of the Arabidopsis *MIR827* promoter. The location of known *cis*-acting elements is shown (for details on *cis*-elements, see Supplemental Table S1).



Supplemental Figure S4. Characterization of *nla* and miR827 OE plants.
A. Structure of the *NLA* gene. The position of the *nla-1* mutation (deletion) is shown. Black bars, untranslated regions; gray bars, coding regions.
B. Pi content (left panel) and accumulation of *NLA* transcripts (right panel) in wild-type and *nla* plants. The statistical significance was determined by *t* test (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$).
C. Accumulation of pre-miR827 and *NLA* transcripts (middle and right panels) and Pi content in miR827 OE plants.
D. Appearance of 3 week-old WT, *nla* and miR827 OE plants.



Supplemental Figure S5. Pi content in wild-type and *nla* plants that have been grown under different Pi supply (0.05 mM, 0.25 and 2 mM Pi).

Supplemental table S1. *cis*-elements identified in the *MIR827* promoter. The nucleotide sequence, copy number (N) and function of each element are indicated.

Cis Element		Sequence	N	Function
SA-related	GT1CONSENSUS	GRWAAW	5	Consensus GT-1 binding site in many light-regulated genes. Binding of GT-1-like factors to the PR-1a promoter influences the level of SA-inducible gene expression.
	WBOXATNPR1	TTGAC	3	"W-box" found in promoter of Arabidopsis thaliana NPR1 gene. The W-box is recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins. Functions in response to environmental stresses.
	ASF1MOTIFCAMV	TGACG	1	"ASF-1 binding site" ASF-1 binds to two TGACG motifs. TGACG motifs are found in many promoters and are involved in transcriptional activation of several genes by auxin and/or salicylic acid.
Pathogen and elicitors-related	WRKY71OS	TGAC	6	Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway. Parsley WRKY proteins bind specifically to TGAC-containing W box elements within the Pathogenesis-Related Class10 (PR-10) genes.
	WBOXNTERF3	TGACY	3	"W box" found in the promoter region of a transcriptional repressor ERF3 gene in tobacco. May be involved in activation of ERF3 gene by wounding.
	BIHD1OS	TGTCA	2	Binding site of OsBIHD1, a rice BELL homeodomain transcription factor. OsBIHD1 is upregulated during disease resistance responses.
	GT1GMSCAM4	GAAAAA	1	GT-1 motif. Plays a role in pathogen- and salt-induced SCaM-4 gene expression.
	MYB1AT	WAACCA	1	MYB1AT, and CGTCA-motifs are pathogen-responsive and hormone-associated cis elements.
	SEBFCONSSTPR10A	YTGTCWC	1	Binding site of the potato silencing element binding factor (SEBF) gene found in promoter of pathogenesis-related gene (PR-10a).
	WBOXNTCHN48	CTGACY	2	NtWRKY1, NtWRKY2 and NtWRKY4; NtWRKYs possibly involved in elicitor-responsive transcription of defense genes in tobacco
	ELRECOREPCRP1	TTGACC	1	ElRE (Elicitor Responsive Element) core of parsley PR1 genes.
PSR-related	P1BS	GNATATNC	1	PHR1-binding sequence found in the upstream regions of phosphate starvation responsive genes from several plant species.

Supplemental table S2. List of oligonucleotides.

Gene name	Accession Number		Sequence (5'-3')
For expression analysis			
β-Tubulin 2	*At5g62690*	Fw	TGTTCAGGCGAGTGAGTGAG
		Rv	ATGTTGCTCTCCGCTTCTGT
Pre-miR399		Fw	TGCATAAATGTTTGTGGTGAGC
		Rv	GAATTACCGGGCAAATCTCCT
Pre-miR827		Fw	ACATGTTGATCATCCTTGTGTTGA
		Rv	CCAAGAAGCGATGCAAAACCA
miR827 stem loop		Rt	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAGTTTG
		Fw	GCGGTTAGATGACCATCAA
NLA	*AT1g02860*	Fw	TGAATTTGTTTGTTGATGGTCA
		Rv	ACAACTCAATAAAACAGATAAAGGT
PR1	*At2g14610*	Fw	GATGTGCCAAAGTGAGGTGTAA
		Rv	GGCTTCTCGTTCACATAATTCC
NPR1	*At1g64280*	Fw	CCGGAAGAGCTTGTTAAAGAGA
		Rv	ATCCGAGTCAAGTGCCTTATGT
ERF1	*At3g23240*	Fw	AACACTCGATGAGACGGAGAAT
		Rv	CTCCCAAATCCTCAAAGACAAC
PDF1.2	*At2g26020*	Fw	CAACAATGGTGGAAGCACAG
		Rv	CTTGCATGCATTGCTGTTTC
MYC2	*At1g32640*	Fw	ATTAATGACCCGATTGGAACAC
		Rv	TGAGCTACCGTTCTCAAACTGA
VSP2	*At5g24770*	Fw	CTCGTCGATTCGAAAACCAT
		Rv	TTCTGCAGTTGGCGTAGTTG
CYP79B2	*AT4g39950*	Fw	GCCGGATATCACATCCCTAA
		Rv	TCCGGTTTAAAGCAAAGTGG
CYP79B3	*AT2g22330*	Fw	CGTGGCACTCTCTGATACGA
		Rv	CAGACCAAACCTTGGGGTTA
CYP71A13	*AT2g30770*	Fw	GGGTAGAGGCTGGACCAAAT
		Rv	ACAACCGAAGATGGAAATGC
CYP71B15	*AT3g26830*	Fw	GGTACGGGATAAATCTCTATGA
		Rv	AGATACAGTCGATGAACCTAC
For fungal DNA quantification			
P.cucumerina_tubulin		Fw	CAAGTATGTTCCCCGAGCCGT
		Rv	GAAGAGCTGACCGAAGGGACC
Ch_ITS2		Fw	AAAGGTAGTGGCGGACCCTC
		Rv	GGCAAGAGTCCCTCCGGAt
Ubiquitin21	*At5g25760*	Fw	AAAGGACCTTCGGAGACTCCTTACG
		Rv	GGTCAAGAATCGAACTTGAGGAGGTT

CHAPTER III

Osa-miR827, a miRNA involved in phosphate starvation response in rice, negatively regulates resistance to the blast fungus *Magnaporthe oryzae*.

Results presented in this chapter are included within a manuscript currently in preparation.

ABSTRACT

Phosphorus is an essential nutrient for plant growth and development. Despite the overall content of phosphorus in soils is generally high, its low bioavailability represents a limiting factor for plant growth. To cope with Pi limitation, plants have developed a robust system to increase Pi uptake, the Phosphate Starvation Response (PSR). The implication of the microRNA miR827 in PSR in Arabidopsis and rice plants is well documented. In Arabidopsis, miR827 targets *NLA* (NITROGEN LIMITATION ADAPTATION) involved in degradation of the plasma-membrane localized PHT1 phosphate transporter. In rice, however, miR827 targets two members of the *SPX-MFS* family of phosphate transporters, *SPX-MFS1* and SPX-MFS2, whose gene products locate at the tonoplast. In this work, we investigated whether miR827 plays a role in disease resistance in rice. We show that CRISPR/Cas9-mediated *MIR827* editing enhances resistance to infection by the fungal pathogen *Magnaporthe oryzae*. This fungus is the causal agent of the blast disease, one of the most damaging diseases of cultivated rice worldwide. Conversely, miR827 overexpression increases disease susceptibility to *M. oryzae*. Compared with wild type plants, the CRISPR/Cas9-edited rice plants had a lower Pi content, while miR827 overexpression caused an increase in Pi content. Alterations in Pi distribution between the vacuoles and the cytoplasm were investigated using ^{31}P-HR-MAS NMR (^{31}P-High Resolution-Magic Angle Spinning Nuclear Magnetic Resonance). No alterations were observed in the vacuolar-to-cytoplasmic Pi ratio between wild type and CRISPR/Cas9-edited rice plants, these plants exhibiting blast resistance. Contrary to this, miR827 overexpressor plants, as well as Pi-treated wild type plants, showed alterations in vacuolar-to-cytoplasmic Pi ratio, which correlated with their phenotype of blast susceptibility. These findings provide evidence for a role of miR827 in resistance to the rice blast fungus *M. oryzae*, while further supporting a link between Pi signaling and disease resistance in rice. Elucidation of miR827 function in rice immunity has a great potential for the development of strategies to improve blast resistance in rice.

INTRODUCTION

Phosphorus (P) is one of the most important macronutrients required for plant growth and development. It is a key component of important molecules and cell structures, such us ATP, nucleic acids, and membrane phospholipids, and also participates in multiple enzymatic reactions and signal transduction cascades (e.g. protein phosphorylation). The plant roots acquire P exclusively in the form of inorganic phosphate (Pi) which is further distributed within the plant. Along with this, multiple inorganic phosphate transporters (PHTs) have been described in plants which are involved in Pi acquisition and allocation (Młodzińska and Zboińska, 2016). Phosphate transporters are grouped into different families and function in different subcellular compartments, such as plasma membrane, tonoplast, chloroplasts or mitochondria (Wang *et al.*, 2017). Despite the fact that total P in soils is relatively abundant, it is one of the less accessible nutrient elements (Hinsinger *et al.*, 2011). P bioavailability is usually low due to rapid conversion into organic and inorganic forms that are not readily available for plant nutrition.

To cope with low Pi supply, plants have developed a robust system to increase Pi uptake and transport, known as the Phosphate Starvation Response (PSR) (Puga *et al*., 2017; Chien *et al*., 2018). During the past years, a considerable progress has been made in characterizing the regulatory mechanisms that underlie PSR in Arabidopsis. A core element of the PSR signaling pathway in Arabidopsis is the *PHOSPHATE STARVATION* RESPONSE1 (PHR1) transcription factor (and related transcription factors) (Nilsson *et al.*, 2007). Under Pi starvation conditions, PHR1 positively regulates the expression of a set of genes involved in PSR, including the microRNAs, miR399 and miR827. The accumulation of these miRNAs substantially increases by Pi deficiency leading to post-transcriptional down-regulation of genes involved in ubiquitination and degradation of Pi transporters. Specifically, miR399 targets *PHOSPHATE2* (*PHO2*) encoding an ubiquitin E2 conjugase implicated in the degradation of the plasma membrane transporter PHT1, as well as the PHOSPHATE 1 (PHO1) transporter (Aung, 2006; Bari *et al.*, 2006; Chiou *et al.*, 2006; Liu *et al.*, 2012). miR827 targets *NITROGEN LIMITATION ADAPTATION* (*NLA*) encoding a RING-type E3 ubiquitin ligase that also mediates the degradation of PHT1 (Lin *et al.*, 2013). In this way, under Pi limiting conditions, *miR399* and miR827 induction relieves negative regulation of Pi transporters for increased Pi uptake in Arabidopsis.

The *OsPHR1*, *OsPHR2* and *OsPHR3* transcription factors (the rice orthologues of *AtPHR1*) also function in the regulation of Pi homeostasis in rice plants (Wang *et al.*, 2012; Guo *et al.*, 2015). A closely related Pi starvation-inducible *PHR* gene, designated as *PHR4*, was also reported to mediate Pi-starvation signaling in rice (Ruan *et al.*, 2017). Remarkably, whereas *miR399* controls *PHO2* expression in both Arabidopsis and rice (Bari *et al.*, 2006), miR827 targets different genes in the two species (Hsieh *et al.*, 2009; Lin *et al.*, 2010a; Lin *et al.*, 2017). As previously mentioned, miR827 targets *NLA* in Arabidopsis (Lin *et al.*, 2013). In rice, however, *miR827* targets transcripts of two vacuolar-located SPX-MFS domain-containing protein genes, namely *OsSPX-MFS1* and *OsSPX-MFS2* (Lin *et al.*, 2010; Lin *et al.*, 2018). The rice SPX-MSF family consists of four members, of which two members (*SPX-MSF1* and *SPX-MSF2*) are regulated by miR827 (Lin *et al.*, 2010). A function in Pi transport across the tonoplast has proposed for SPX-MSF proteins (Secco *et al.*, 2012).

In plants, vacuoles serve as a primary Pi reservoir and have a dual role as both sink and source of Pi, thus, helping the plant to maintain cellular Pi homeostasis. Compared to plasma membrane transporters, however, the function of vacuolar Pi transporters is far less characterized. The Arabidopsis PHT5;1 (also known as VPT for Vacuolar Phosphate Transporter 1) is known to function as a major vacuolar Pi influx transporter (Liu *et al.*, 2015; Liu *et al.*, 2016). Given that *OsSPX-MFS1* complements the phenotype of the Arabidopsis *pht5;1* mutant, it was proposed that the rice SPX-MFS1 might function as a vacuolar Pi influx transporter (Lin *et al.*, 2010; Liu *et al.*, 2016). In rice, however, the situation is more complex than that in Arabidopsis, and less is known about the interplay of the different vacuolar transporters in the regulation of Pi distribution within cellular compartments, in particular between the vacuoles and cytoplasm. In previous studies, it was described that *OsSPX-MSF1* and *OsSPX-MSF2* (the miR827 target genes) are regulated in an opposite manner during Pi starvation in rice plants (Lin *et al.*, 2010). Whereas *OsSPX-MFS1* expression is suppressed by Pi deficiency, *OsSPX-MFS2* is induced (Lin *et al.*, 2010; Wang *et al.*, 2012). Clearly, the regulation of *SPX-MSF1* and *SPX-MFS2,* appears to be governed by complex regulatory mechanisms in the rice response to Pi starvation.

On the other hand, it is generally believed that deficiency or excess of nutrients, in particular Pi, might have an impact on disease resistance in plants (Castrillo *et al.*, 2017; Chan *et al.*, 2021). The current scenario is that plant/pathogen interactions might have different outcomes depending not only on Pi availability, but also on the interacting partners, host plant and type of pathogen.

In this work, we investigated whether miR827, and miR827-mediated alterations in Pi content, has an effect on disease resistance in rice. We show that MIR827 editing by the CRISPR/Cas9 technology causes a reduction in Pi content, while increasing resistance to infection by the fungal pathogen *Magnaporthe oryzae*. Contrary to this, miR827 overexpression causes an increase in Pi content and enhances susceptibility to *M. oryzae* infection. The fungus *M. oryzae* is the causal agent of the rice blast disease, one of the most devastating disease of cultivated rice worldwide (Wilson and Talbot, 2009; Fernandez and Orth, 2018). The distribution of vacuolar and cytosolic Pi content has been investigated in intact leaves of CRISPR-Cas9-edited and *MIR827* overexpressor rice plants using ^{31}P-HR-MAS NMR (^{31}P-High Resolution-Magic Angle Spinning Nuclear Magnetic Resonance). Collectively, results here presented support a role of miR827 in controlling blast resistance in rice, most probably, by modulating Pi content and Pi distribution between subcellular compartments. This study broadens our knowledge about miRNAs playing a role in rice immunity while identifying interconnected regulations between Pi homeostasis and disease resistance in rice. This information will be useful in designing strategies to protect rice plants against pathogen infection.

EXPERIMENTAL PROCEDURES

Plant materials, genotyping and growth conditions

Overexpressor miR827 rice (cultivar Nipponbare) plants kindly provided by Dr. Jen Chiou (Academia Sinica, Taipei). The miR827 rice mutants (cv. Nipponbare) were produced by Agrobacterium-mediated transformation of embryogenic calli derived from mature embryos (Sallaud *et al*., 2003) through CRISPR-Cas9 technology, using the multiplexed system described by Lowder *et al*. 2015. Two sgRNAs were designed to target the MIR827 precursor using CRISPR-P 2.0 design tool (Liu *et al.*, 2017). The sgRNAs were annealed and cloned to vectors pYPQ131D and pYPQ132D. and then transferred to pYPQ142 vector by digestion and ligation, where the guides got under the control of *OsU3* promoter. pYPQ142 vector was assembled with pYPQ167 (containing the *Cas9* gene) in the pYPQ203 T-DNA transformation vector by gateway recombination. For genotyping, genomic DNA was obtained as described (Murray and Thompson, 1980), but using MATAB as extraction buffer (0.1 M Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% MATAB, 1% PEG 6000 and 0.5% sodium sulphite). CRISPR-derived mutations were screened by PCR using the primers listed in **Supplemental table S1**.

Plants were grown in soil at 28 ºC with a 14 hr:10 hr light/dark cycle. In vitro plants were grown during one week in water and a second week in Murashigue and Scoog medium at 0.3, 0.6, 1.25, 2.5 and 5 mM of KH_2PO_4 for phosphate treatment.

^{31}P-NMR analysis of vacuolar and cytoplasmic Pi.

Rice plants were grown *in vitro* in Murashigue and Skoog media. For wild type plants grown under different Pi supplies, plants were grown in Murashigue and Skoog containing different concentrations of KH_2PO_4 (0.25mM, 0.5mM and 2.5mM). Plants were grown for two weeks at 28 ºC with a 14 hr:10 hr light/dark cycle. ^{31}P-HR-MAS **(**^{31}P-High Resolution-Magic Angle Spinning Nuclear Magnetic Resonance) analyses were carried out at the NMR Facility of the Autonomous University of Barcelona (UAB) (https://sct.uab.cat/sermn/), which is also part of NANBIOSIS ICTS, Unit 25

(https://www.nanbiosis.es/portfolio/u25-nmr-biomedical-application-i/) by using the 400MHz-Advance III machine. The analysis was performed in the second leaf.

Free phosphate content in rice plants

The free Pi content in rice plants was determined as previously described (Ames, 1966; Versaw and Harrison, 2002). Rice leaves were frozen in liquid nitrogen and grounded. The powder obtained was treated with 1% glacial acid acetic. A mix of ammonium molybdate and ascorbic acid was added to the solution producing a blue color more or less intense depending on the phosphate concentration. Free Pi content was determined measuring the absorbance at 820nm.

Infection assays

For infection assays, the fungus *M. oryzae* (strain Guy 11) was grown on 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. Soil-grown rice plants at the three- to four-leaf stage were spray inoculated with a spore suspension of *M. oryzae* adjusted at the desire concentration. The percentage of diseased leaf area was determined at 4 dpi by using the APS Assess 2.0 program (Lakhdar, 2008). Fungal biomass was quantified by quantitative PCR (qPCR) using specific primers for the *M. oryzae 28S* DNA gene (Qi and Yang, 2002).

Gene expression analyses

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from TURBO DNAse (Ambion) treated total RNA (1 µg) with High Capacity cDNA reverse transcription kit (Applied Biosystems) and oligo-dT. Reverse transcription (RT)-qPCR analysis were performed in optical 96-well plates, in a Light Cycler 480, using SYBR® green (Roche). Primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The *Ubiquitin1* gene

(Os06g0681400) was used to normalize the transcript level in each sample. At least three independent biological replicates with three technical replicates were analysed. Accumulation of mature miR827 was determined by stem-loop reverse transcription quantitative PCR (Varkonyi-Gasic *et al*., 2007). Primers used in this study are listed in **Supplemental Table 1**.

RESULTS

MiRNAs represent a class of small, non-coding RNAs that post-transcriptionally repress gene expression through sequence complementarity to target transcripts (Bartel, 2004). The function of miRNAs in controlling developmental programs and executing responses to biotic/abiotic cues, is well documented (Jeong and Green, 2013; Staiger *et al.*, 2013; Weiberg *et al.*, 2014). miRNAs are synthesized as long precursor molecules (primary miRNAs, pri-miRNAs) that are processed in a two-step process by a DICER-Like (DCL) ribonuclease, to produce shorter precursor miRNA molecules (pre-miRNA) and miRNA-5p/miRNA-3p duplex. Finally, the functional strand of the mature *miRNA duplex* is incorporated into the RNA-induced silencing complex (RISC) to guide cleavage or translational inhibition of target transcripts (Rogers and Chen, 2013). The miRNA registry, or miRBase, is a widely used database for known miRNAs (https://www.mirbase.org).

According to miRBase (release 22), the miR827 family contains 3 members, registered as miR827 (henceforth miR827a), miR827b and miR827c. We noticed that the mature miR827a and miR827b sequences are identical, with only a single nucleotide difference at their 5' terminal position, a typical feature of miRNA isoforms or isomiRs (**Figure 1A**) (Fard *et al.*, 2020). Moreover, the nucleotide sequences of the miR827a and miR827b precursors are identical (**Figure 1B**). As for miR827c, its mature and precursor sequences differ from the miR827a/miR827b precursor sequences (**Figure 1A, B**).

A

```
osa-miR827a       -UUAGAUGACCAUCAGCAAACA      21
osa-miR827b       GUUAGAUGACCAUCAGCAAAC-      21
                   ********************

osa-miR827a       UUAGAUGACCAUCAGCAAACA       21
osa-miR827c       UUAGAUGACCAUCAGCGAAAA       21
                  ****************.**.*
```

B

```
osa-MIR827a   -----------UUUUGUUGCUGGUCAUCUAGCUACCCGUGCAUGCCUGGAGAUUGGAGAA  49
osa-MIR827b   CCAUGAACCUGUUUUGUUGCUGGUCAUCUAGCUACCCGUGCAUGCCUGGAGAUUGGAGAA  60
                         *********************************************

osa-MIR827a   UAAUUGACGAUGCAGCAGUCGGCUUAUUGGCUCUUGGGCACGCGUGGUUAGAUGACCAUC 109
osa-MIR827b   UAAUUGACGAUGCAGCAGUCGGCUUAUUGGCUCUUGGGCACGCGUGGUUAGAUGACCAUC 120
              ************************************************************

osa-MIR827a   AGCAAACA----------  117
osa-MIR827b   AGCAAACAAGUUCGUGAG  138
              ********
```

```
osa-MIR827a   ---UUUUGUUGCUGGUCAU------CUAGCUACCCGUGCAUGCCUGGAGAUUGGAGAAUA  51
osa-MIR827c   GAUUUUCGCUGACGGCCGAUUUAACACAGCCACCAGCGAA---------AAUGAA---UU  48
                 *** * **. ** *.       . *** ***.* *.*         * **.*   *

osa-MIR827a   AUUGACGAUGCAGCAGUCG--------------GCUUAUUGGCUCUU-------GGGCAC  90
osa-MIR827c   UUUG--CUAGCGGCUGAUGUAAGAUGACCGCUAGCAAAGAUCCAUUUUUGCUGGUGGCUG 106
               ***     **.** *  *              **  *    *  **        ***

osa-MIR827a   GCGUGGUUAGAUGACCAUCAGCAAACA--   117
osa-MIR827c   GC---UUAAGAUGACCAUCAGCGAAAAUG   132
              **    * **************.**.*
```

Figure 1. Nucleotide sequences of miR827 family members, mature and precursor sequences, as registered in miRBase.
A. Alignment of mature miR827 sequences, miR827a (referred to as miR827 in miRBase), miR827b and miR827c.
B. Alignment of miRNA precursor sequences. Mature miR827 sequences are indicated by black bars.

On the other hand, the mature miR827 sequence (as annotated in miRBase) maps to the 3'end of the stem region, a feature that does not meet the requirements for miRNA precursor processing (**Figure 2A**). Structural determinants needed to guide miRNA precursor processing include a miRNA-5p/miRNA-3p duplex being located within a stem region that also contains a lower and upper stem region (Bologna *et al.*, 2013; Rojas *et al.*, 2020). By extending the nucleotide sequence of the genomic region surrounding the

MIR827a locus (20 nucleotides upstream/downstream), a stem-loop hairpin structure was predicted in which the mature miR827 sequence maps within a longer stem region (**Figure 2B**). Likely, the existing annotation of miR827 species in rice needs to be revised. The rice miR827 family most probably consists of two family members, miR827a (miR827 and miR827b in the current miRBase release) and miR827b (miR827c in miRBase).

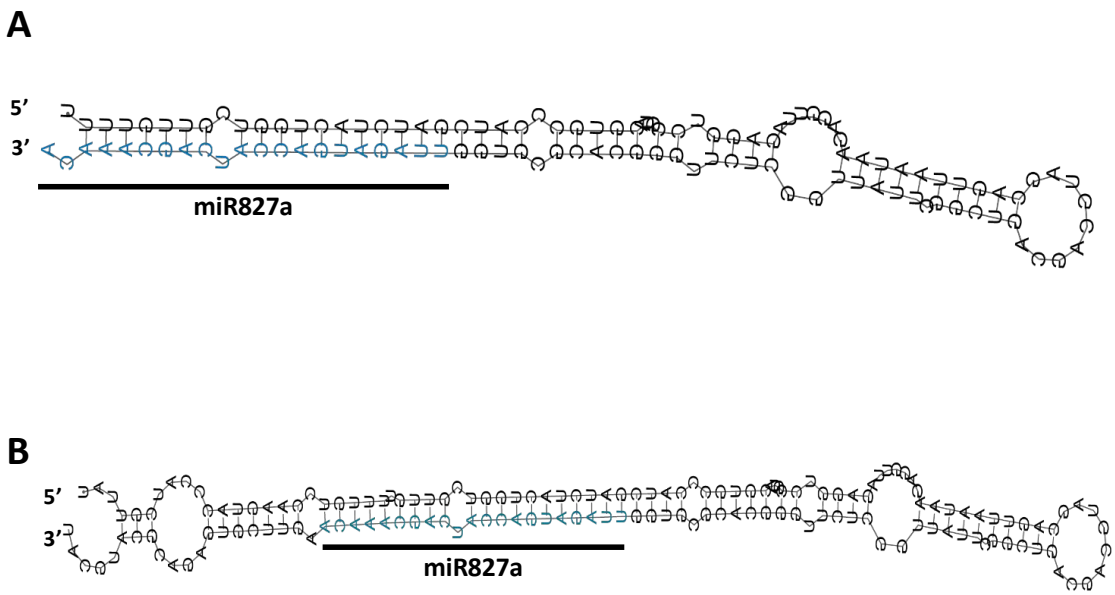


Figure 2. Precursor structure of miR827.
A. Fold-back structure currently registered in miRBase (https://www.mirbase.org; release 22)
B. Fold-back structure of the miRNA precursor in which the nucleotide sequence has been extended relative to the annotated sequence in the miRBase (20 nucleotides in the 5' and 3' direction).

CRISPR/Cas9-mediated mutagenesis of *MIR827* enhances resistance to infection by *M. oryzae*

Typically, the functional characterization of a *MIRNA* requires the use of gain- and loss-of function approaches. However, because of the small size of *MIR* genes, identifying mutant alleles for miRNAs in insertional mutant collections is unlikely. Alternative techniques have been developed to study miRNA function, such as interference with miRNA activity through target mimicry. Nowadays, the CRISPR/Cas9 (clustered regulatory interspaced short palindromic repeat-associated nuclease 9) technology

opens new possibilities to knock-out *MIR* genes, hence, for the functional characterization of miRNAs of interest.

In this work, the CRISPR/Cas9 system was used to create targeted deletions in the miR827 precursor sequence. For this, two single-guide RNA (sgRNA) were designed based on the presence of two PAM (protospacer adjacent motif, UGG) sequences located inside the precursor sequence (**Figure 3A**). Transgenic rice (*O. sativa* cv Nipponbare) plants were produced by *Agrobacterium*-mediated transformation. The resulting hygromycin-resistant rice lines were screened by PCR using gene-specific primers located upstream and downstream of the target site. **Figure 3A,** shows genomic deletions in the miR827 precursor identified in T0 plants. The CRISPR/Cas9-induced indels were confirmed by DNA sequencing. Mutations induced by CRISPR/Cas9 in T2 homozygous plants consisted of a deletion of 88, nucleotides (line Δ88) and deletion and insertion of 48 and 7 nucleotides respectively (line 7Δ48) (**Figure 3A**).

These lines showed large indels in the pre-miR827 precursor sequence, namely lines 38.7 (Δ48/+7) and 23.5 (Δ88). We reasoned that these deletions may disrupt miRNA precursor structures, hence miRNA precursor processing and function. Along with this, *MIR827* expression was abolished in the CRISPR/Cas9-edited miR827 plants grown under low Pi supply (**Figure 3B**).

Of the two miR827 target genes, *OsSPX-MSF1* and *OsSPX-MSF2*, only *SPX-MFS1* was found to be up-regulated in CRISPR/Cas9-edited rice plants (**Figure 3B**). In contrast, *SPX-MSF2* expression was not significantly different in CRIPR/Cas9-edited plants compared with wild-type plants (**Figure 3B**). Here, it is worth mentioning that while *OsSPX-MSF1* expression is known to be repressed by Pi starvation, the expression of *OsSPX-MSF2* increases in Pi-starved rice plants (Lin *et al.*, 2010). Our results indicated that growing the CRISPR/Cas9-edited miR827 rice plants under low Pi supply results in higher accumulation of *SPX-MSF1* transcripts, while the Pi starvation-inducible expression of *SPX-MSF2* in these plants is compromised (**Figure 3B**). No phenotypic differences were

visible among the CRISPR-miR827lines (homozygous T2 plants) and wild-type plants when grown under low Pi or sufficient Pi supply (**Figure 1A**).

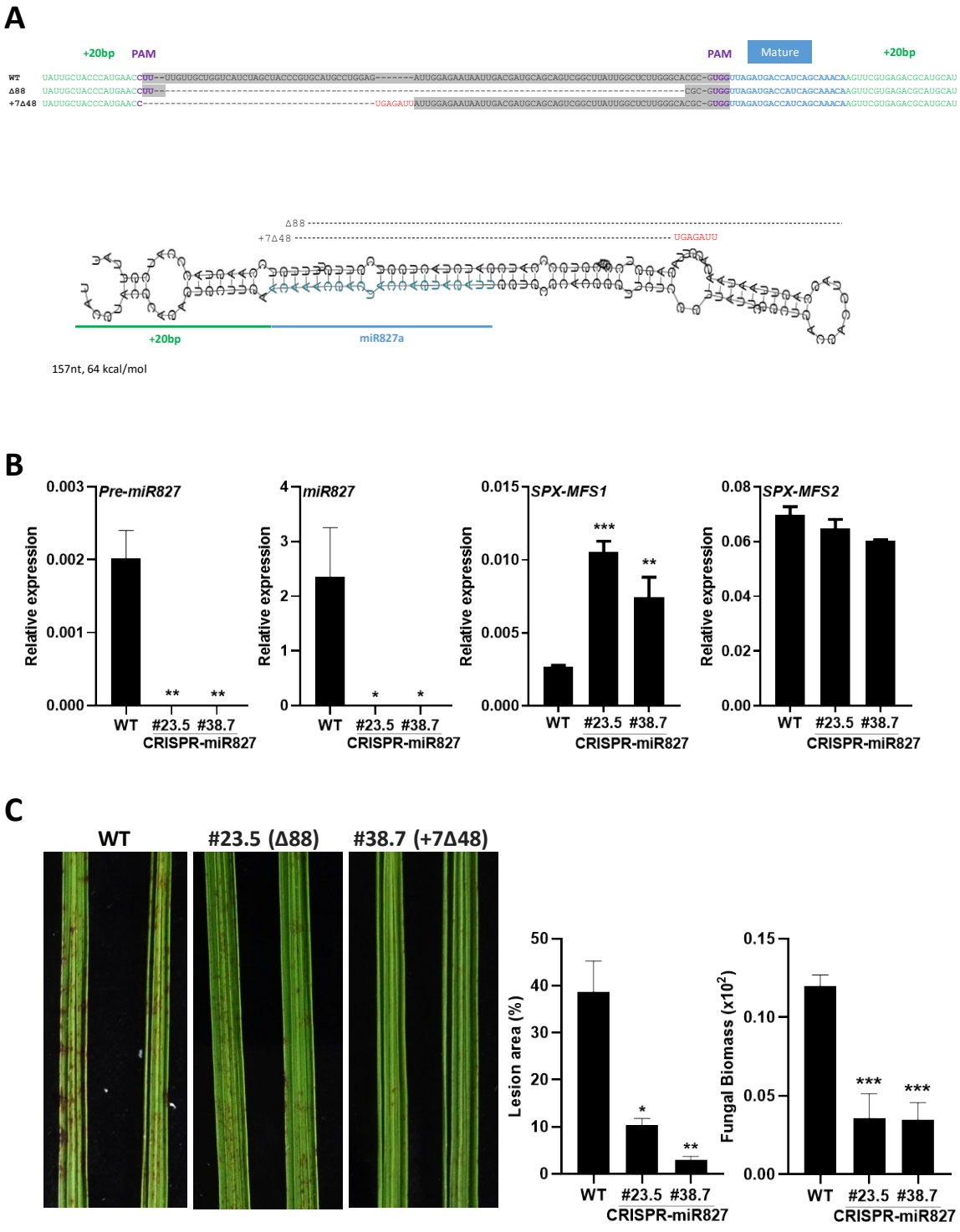


Figure 3. CRISPR/Cas9-induced mutations in the *MIR827* gene confers resistance to *M. oryzae* infection. Statistical significance in B and C was determined by ANOVA (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$).

A. Mutations generated by CRISPR/Cas9 editing in the miR827 precursor sequence are shown in the upper part of the precursor structure. Two alleles were selected: Δ88 and Δ48/+7. Dashes

indicate deletions. In red, 7 nt insertion. The PAM motifs (UGG) are indicated. The secondary structure of the extended miR827 precursor was predicted using RNAfold. The length of the precursor (nt) and the minimum free energy of folding (MFE, kcal/mol) are indicated.
B. Accumulation of miR827 precursor (pre-miR827), mature miR827, and SPX-MFS1 and SPX-MSF2 transcripts was determined by RT-qPCR (pre-miR827, SPX-MSF1, SPX-MSF2) and Stem-loop quantitative reverse transcription PCR (stem loop RT-qPCR).
C. Representative images of *M. oryzae*-infected CRISPR/Cas9-edited miR827 plants at 5 days post-inoculation (dpi). Right panel, quantification of lesion area and fungal biomass. Quantification of blast lesions was carried out by image analysis. Relative quantification of fungal biomass was determined by qPCR using specific primers for *M. oryzae* 28S DNA. Of three technical/biological replicates of fungal DNA levels normalized against the rice *Ubiquitin 1* gene. Results are from one out of three independent experiments (8 plants/genotype), which gave similar results.

To investigate the functional relevance of *MIR827* mutations in disease resistance, CRISPR-miR827 lines (hereinafter CRISPR-miR827 plants; lines #23.5 and #38.7) and wild-type plants were grown under low Pi supply for 3 weeks and then they were spray-inoculated with *M. oryzae* spores. The CRISPR-miR827 plants consistently showed resistance to *M. oryzae* infection as revealed by visual inspection, quantification of the lesion area and fungal biomass in leaves of CRISPR-miR827 and wild-type plants (**Figure 3C**)**.** From these results it can be concluded that mutations generated in the miR827 precursor using CRISPR/Cas9 prevents the accumulation of mature miR827 species, and that *MIR827* knockout mutations is accompanied by resistance to infection by the blast fungus *M. oryzae*.

MIR827 overexpression increases susceptibility to *M. oryzae* infection

To further investigate whether miR827 plays a role in disease resistance in rice, we generated MIR827 overexpressor (miR827 OE) rice plants. The transgenic miR827 OE plants accumulated pre-miR827 transcripts (**Figure 4A**). Regarding miR827 target genes, both *SPX-MFS1* and *SPX-MFS2* expression was found to be down-regulated in miR827 OE lines (**Figure 4A**). No phenotypic differences were observed in miR827 OE respect to wild-type plants when grown under low Pi or sufficient Pi supply (**Figure S1B**).

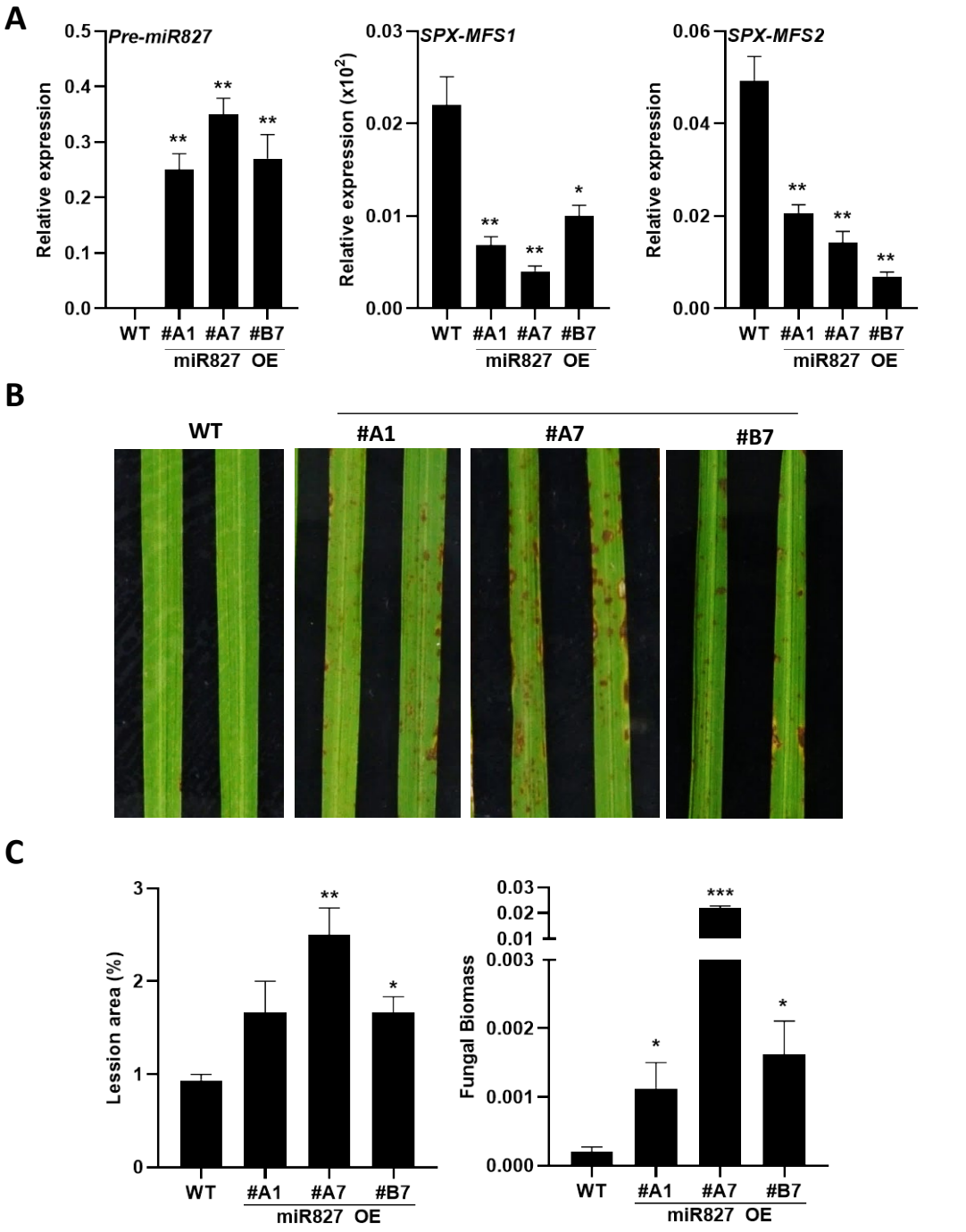


Figure 4. Susceptibility to infection by the rice blast fungus *M. oryzae* in miR827 overexpressor rice plants (miR827 OE).

A. *pre-miR827, SPX-MSF1* and *SPX-MSF2.*

B. Susceptibility of miR827 OE plants to *M. oryzae* infection.

C. Quantification of blast lesions was carried out by image analysis (left panel). Relative quantification of fungal biomass was determined by qPCR using specific primers for *M. oryzae* 28S DNA (values are fungal DNA levels normalized against the rice *Ubiquitin 1* gene (right panel). Bars represent mean ± SEM (n = 8) (*One Way ANOVA*, $^{**}p \leq 0.05$ and $^{**}p \leq 0.01$).

Infection assays with *M. oryzae* were carried out with three independently generated miR827 OE lines. At 7 dpi, leaves of miR827 OE plants were severely affected by blast lesions whereas, by the same time of infection, leaves of *M. oryzae*-infected wild type showed less blast symptoms (**Figure 4B**). Quantification of lesion area and fungal biomass confirmed enhanced susceptibility to infection by the rice blast fungus in miR827 OE plants compared with wild-type plants. The same results were observed at the T2 and T3 generations.

Collectively, this study revealed a phenotype of resistance or susceptibility to *M. oryzae* infection in CRISPR-miR827 and miR827 OE plants, respectively, thus, supporting the involvement of miR827 in blast resistance in rice.

Pi homeostasis is perturbed in CRISPR/Cas9 edited and miR827 overexpressor rice plants

To assess whether there is a relationship between disease phenotype and Pi content as a result of alteration in *MIR827* expression, we determined the Pi content (e.g. soluble Pi) in wild-type, miR827 OE and CRISPR-miR827 plants grown under low Pi conditions, a condition in which miR827 accumulation is compromised in CRISPR-miR827 plants. That the CRISPR-miR827 plants perceive and respond to low-Pi supply was confirmed by examining *OsPHR4* expression, whose expression is known to be induced by Pi starvation in rice (Ruan *et al.*, 2017). As shown in **Figure S2**, *OsPHR4* expression was up-regulated in CRISPR-miR827 plants grown under low Pi supply.

Compared with wild-type plants, miR827 OE plants were found to accumulate higher levels of Pi in leaves and roots (**Figure 5A**). Conversely, CRISPR-miR827 plants had reduced Pi levels (**Figure 5A**), thus susceptibility and resistance phenotype to *M. oryzae* infection in miR827 OE and CRISPR-miR827 coincide with increased and decreased Pi content, respectively. Further supporting a relationship between Pi accumulation and blast susceptibility, we previously reported that miR399 overexpressor lines, which also overaccumulate Pi, showed blast susceptibility (Campos-Soriano *et al.*, 2020).

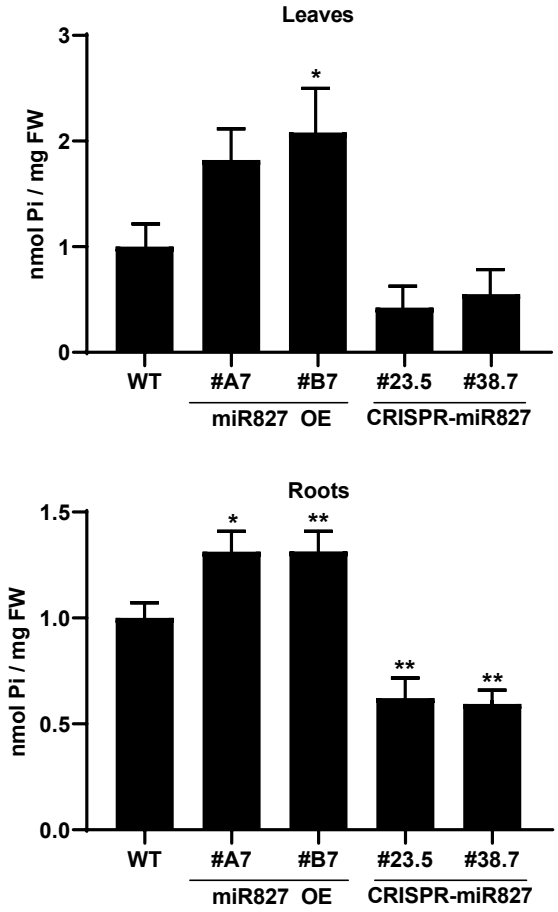


Figure 5. Pi content in leaf and root tissues of CRISPR-miR827 and miR827 OE plants grown with low Pi. Soluble Pi content was determined as previously described (Versaw and Harrison, 2002). The Pi level in wild-type plants was set to 1.0. For each line, the values represent fold changes in the accumulation of Pi relative to wild-type plants. Values shown are the mean ± SEM of four biological replicates and three plants per replicate (*t-test*, *$p \leq 0.05$ and **$p \leq 0.01$)

Knowing that miR827 targets *SPX-MFS* genes, potentially contributing to maintain Pi balance between cytoplasmic and vacuolar Pi pools, we hypothesized that miR827 overexpression and/or CRISPR/Cas9-mediated mutagenesis of *MIR827* might provoke alterations in Pi distribution between the vacuole and cytoplasm. In this work, the subcellular distribution of Pi (e.g. vacuoles and cytoplasm) was examined on intact rice leaves using ^{31}P-Magnetic resonance spectroscopy (^{31}P-MRS). In previous studies, ^{31}P-MRS was successfully applied to the analysis of vacuolar and cytoplasmic Pi (from now on vac-Pi and cyt-Pi, respectively) in Arabidopsis plants (Liu *et al.*, 2016). In our hands, however, ^{31}P-MRS did not yield good spectra resolution when applied to rice. Contrary

to this, chemical shifts from vacuolar and cytoplasmic Pi were well resolved using *in vivo* ^{31}P-HR-MAS NMR (^{31}P-High Resolution-Magic Angle Spinning Nuclear Magnetic Resonance).

Before analyzing Pi levels in miR827 OE and CRISPR-miR827 plants, we assessed whether treatment of rice plants with Pi results in alterations in vacuolar and cytoplasmic Pi content. In the range of Pi concentrations assayed in this study (0.25 mM, 0.5 mM, 2.5 mM Pi), a tendency to a higher vacuolar Pi content was observed when increasing Pi supply (**Figure 6A,** left panel). There is still the possibility that higher Pi concentrations or longer periods of Pi treatment are needed to observe significant differences in vacuolar Pi content in Pi-treated rice leaves. On the contrary, accumulation of cytoplasmic Pi gradually increased as Pi concentration increased (**Figure 6A**, middle panel). Accordingly, the vac-Pi/cyt-Pi ratio decreased in response to Pi treatment in wild type rice plants, with vac-Pi/cyt-Pi values of 5.8 ± 0.7, 4.2 ± 1.3 and 2.5 ± 0.6 at 0.25 mM, 0.5 mM and 2.5 mM Pi, respectively (**Figure 6A**, right panel). In parallel, we measured total Pi level of the plants used for ^{31}P-HR-MAS analysis. Increases in Pi supply correlated well with an increase in total Pi content in wild-type plants (**Figure S3A**).

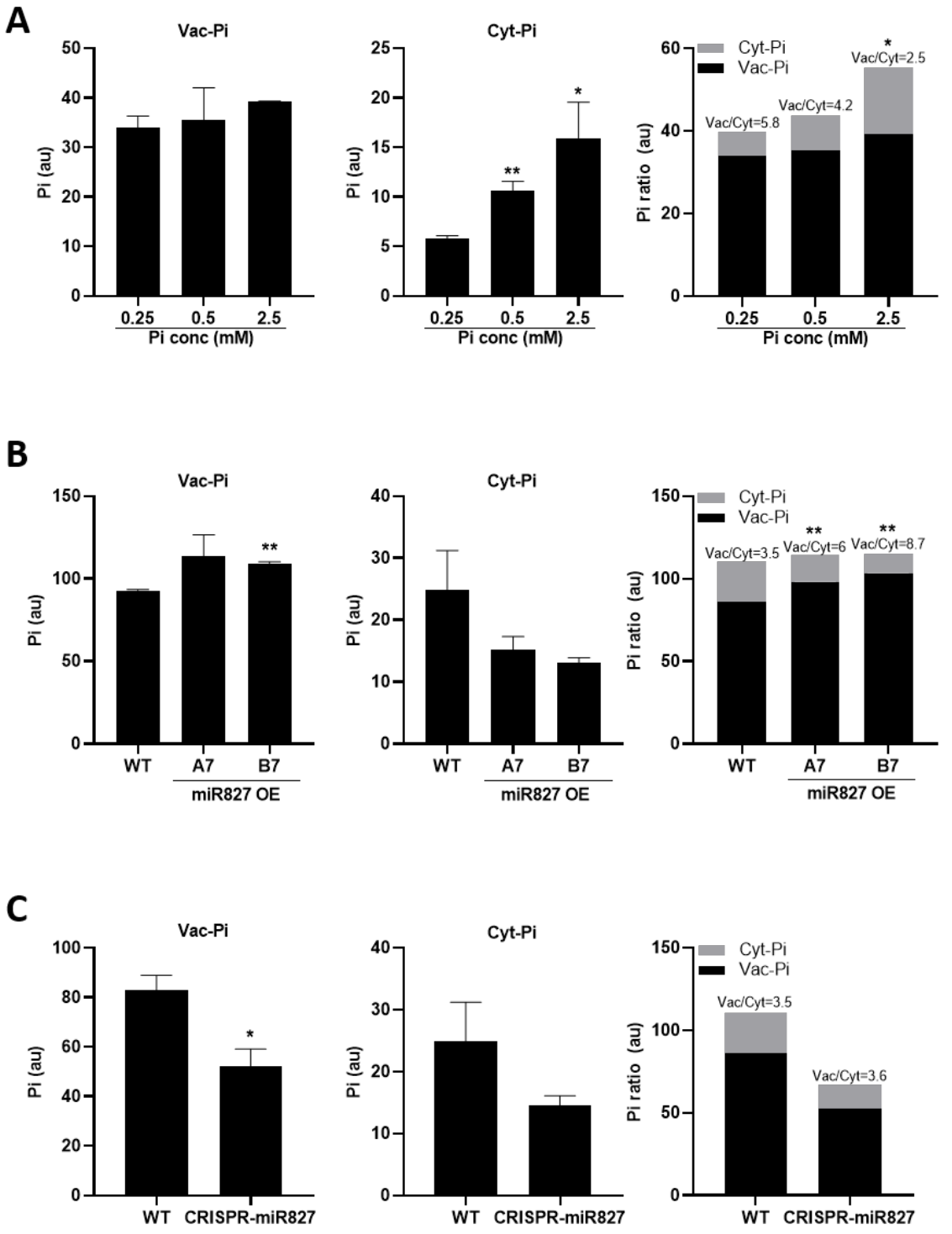


Figure 6. Vacuolar and cytoplasmic Pi content in Pi-treated, miR827 and CRISPR-miR827 rice plants. Pi distribution between the vacuole and the cytoplasm, and Pi ratio between vac-Pi and cyt-Pi is presented. The Pi content was determined on intact rice leaves by ^{31}P-HR-MAS. Two independent experiments were carried out with at least 5 plants per experiment. Values represent arbitrary units (au) (*t* test, *$p \leq 0.05$ and **$p \leq 0.01$).

A. Vacuolar (Vac-Pi) and cytosolic Pi (Cyt-Pi) levels in rice plants grown under increasing concentrations of Pi (0.25 mM, 0.5 mM and 2.5 mM). Representation of Vac-Cyt Pi distribution. Asterisks indicate significant differences in the Vac-Pi/Cyt-Pi relative to the lowest Pi concentration (0.25mM).

B. Vacuolar and cytosolic Pi in miR827 OE (lines A7 and B7) (left and middle panels). Right panel, representation of Vacuolar-to-Cytoplasmic Pi distribution. Asterisks indicate significant differences in the Vac-Pi/Cyt-Pi ratio between miR827-OE and wild-type plants.
C. Vacuolar and cytosolic Pi in CRISPR-miR827 (line #38.7) (left and middle panels). Right panel, representation of the vacuolar-to-cytoplasmic Pi ratio.

Next, we examined Pi distribution between the vacuoles and the cytoplasm of miR827 OE and CRISPR-miR827 plants using ^{31}P HR-MAS. Compared with wild-type plants, miR827 OE plants had an increase in vacuolar Pi content while the cytoplasmic Pi level in these plants was reduced (**Figure 6B,** left and middle panels). The vac/cyt Pi ratio in miR827 OE plants was calculated as 6 ± 0.9 and 8.7 ± 1.3 (lines A7 and B7, respectively), whereas wild-type plants had a vac/cyt Pi ratio of 3.5 ± 0.9 (**Figure 6B**, right panel). On the other hand, the vacuolar Pi content in CRISPR-miR827 plants was reduced compared with wild-type plants (**Figure 6C**). Intriguingly, the CRISPR-miR827 plants also had reduced cytosolic Pi level relative to wild-type plants. As a consequence, the vac/cyt Pi ratio was not altered in CRISPR-miR827 (3.5 ± 0.9 and 3.6 ± 0.6 in wild type and CRISPR-miR827, respectively). Total Pi levels were also measured in this condition in which miR827-OE plants had a higher Pi content compared with wild type plants (**Figure S3B**). From these results it is concluded that Pi treatment and miR827 overexpression is accompanied by alterations in the vac/cyt Pi ratio and blast susceptibility. In contrast, in the CRISPR-miR827 plants, the vac/cyt Pi ratio is not affected, and these plants exhibit blast resistance. Presumably, the CRISPR/Cas9 plants must orchestrate a set of Pi transport mechanisms to maintain Pi homeostasis at the cellular level under low Pi conditions. If so, it can be hypothesized that maintenance of cellular Pi homeostasis is important factor for blast resistance in rice.

DISCUSSION

It has long been recognized that plant miRNAs function as master regulators of gene expression in diverse developmental processes (Mallory and Vaucheret, 2006; Li *et al.*, 2017). However, much less is known about the role of miRNAs in plant immunity, perhaps because most studies on plant defense against pathogens focused on transcriptional reprograming, rather than post-transcriptional reprogramming of gene expression. A further consideration regarding miRNAs is that usually these riboregulators function in fine-tuning of gene expression, instead of turning genes on and off. This fact makes miRNAs perfectly suited for modulation of signaling pathways and gene regulatory networks. Nevertheless, our understanding of miRNA-mediated processes underlying disease resistance in plants under nutrient stress conditions is far from complete.

In this study, we provide evidence that, in addition to being an important component in Pi signaling, miR827 negatively regulates blast resistance in rice. Several pieces of data support this conclusion. First, CRISPR/Cas9-mediated *MIR827* editing enhances blast resistance, while miR827 overexpression increases disease susceptibility. Second, previous studies indicated that overaccumulation of Pi in rice enhances blast susceptibility (Campos-Soriano *et al.*, 2020). In line with this, Pi accumulation in mmiR827 OE results in blast susceptibility, whereas a reduction in Pi content confers resistance to *M. oryzae* infection in CRISPR-miR827 plants. Finally, although it does not rigorously prove, small RNA sequencing analysis showed regulation of miR827 expression during *M. oryzae* infection or treatment with *M. oryzae* elicitors (Campo *et al.*, 2013; Li *et al.*, 2014). This piece of evidence supports a role of miR827 in rice immunity. The exact mechanisms by which miR827 modulates immune responses deserves further investigation.

The observation that silencing and overexpression of *MIR827* has an impact on disease resistance raised an interesting question related to the disease phenotype that is observed in rice and Arabidopsis growing under different Pi regimes. Whereas Pi overaccumulation increases susceptibility to pathogen infection in rice (Campos-Soriano *et al.*, 2020; Chapter III in this PhD Thesis), in Arabidopsis the accumulation of Pi results in resistance to pathogen infection (Chapters I and Chapter II, this PhD Thesis). Different

explanations, which are not mutually exclusive, might explain these differences. In addition to host factors, resistance or susceptibility might be dependent on the type of pathogen that infects rice and Arabidopsis plants. In Arabidopsis, this is improbable, as we could observe resistance to infection with necrotrophic and hemibiotrophic fungal pathogens (*P. cucumerina* and *C. higginsianum*, respectively). In the case of rice, however, only one pathogen with a hemibiotrophic lifestyle (*M. oryzae*) was used in infection experiments. Further research using other types of pathogens will help in explaining differences between rice and Arabidopsis in terms of resistance or susceptibility in relation to Pi supply. Alternatively, differences in the molecular mechanisms by which Arabidopsis and rice plants integrate Pi signaling and immune responses might explain different disease phenotypes caused by Pi overaccumulation in the two plant species. In favor of this possibility, miR827 is known to target different genes in rice and Arabidopsis plants, which already points to different regulatory mechanisms controlling Pi homeostasis. Further supporting a link between miR827-mediated mechanisms controlling disease resistance in Arabidopsis, the miR827/*NLA* regulatory module was previously found to be involved in resistance of Arabidopsis to the bacterial pathogen *Pseudomonas syringae* DC3000, while enhancing susceptibility to the cyst nematode *Heterodera schachtii* (Yaeno and Iba, 2008.; Hewezi *et al.*, 2016). Cross-talk between Pi signaling and immune signaling might then regulate disease resistance to different pathogenic organisms.

Not only total Pi content, but also the subcellular distribution of Pi (e.g. between vacuoles and cytoplasm) might explain resistance to pathogen infection in rice. Thus, we show that alterations in the vac/cyt Pi ratio is associated to a phenotype of blast susceptibility. This effect was observed in miR827-OE plants (higher vac/cyt Pi ratio than wild type plants) and wild-type plants grown under high Pi supply (lower vac/cyt Pi ratio compared to wild type plants grown under low Pi supply). Remarkably, CRISPR-miR827 plants do not exhibit alterations in the subcellular distribution of Pi, these plants showing pathogen resistance. This observation highlights the relevance of homeostatic regulation of Pi in CRISPR/Cas9-edited miR827 plants.

Maintenance of Pi homeostasis at the cellular level might be an important factor in determining the outcome of a particular plant/pathogen interaction, resistance or

susceptibility. Thus, although the total Pi content is reduced in CRISPR/Cas9 miR827 plants compared with wild-type plants, the vacuolar-to-cytoplasmic Pi ratio is not altered in these plants. Regarding the miR827 target genes, *SPX-MSF1* and *SPX-MSF2*, it was reported that these genes respond in an opposite manner to Pi supply (down- and up-regulated by Pi starvation, respectively) (Lin *et al.*, 2010). On the other hand, the Pi transport activity of SPX-MSF proteins as vacuolar Pi influx/efflux transporters is still unclear. While it is generally accepted that SPX-MSF1 mediates Pi influx into the vacuole (Wang *et al.*, 2012), whether SPX-MFS2 mediates influx or efflux of Pi between the vacuoles and the cytoplasm remains unclear. For SPX-MFS3, which is also a tonoplast-localized protein, a Pi efflux activity from the vacuole to the cytosol was proposed (Wang *et al.*, 2015). It is also true that, whereas SPX-MSF3 localizes at the tonoplast in rice, heterologous expression of *OsSPX-MFS3* in yeast and oocytes showed a plasma membrane localization of SPX-MFS3, this transporter facilitating influx or efflux of Pi depending on the external Pi concentration (Wang *et al.*, 2015). On this basis, at this moment, there is not an obvious explanation about mechanisms that orchestrate Pi transport between the cytoplasm and vacuoles in CRISPR/Cas9 plants. Because *SPX-MSF1* expression is up-regulated in CRISPR/Cas9 plants, an increase in Pi concentration in the vacuole would be expected, but the vac/cyt Pi ratio was not altered in these plants. It is tempting to hypothesize that loss-of-function of *MIR827* and up-regulation of *SPX-MSF1* might activate mechanisms of compensation implicating Pi transporters other than the miR827-regulated *SPX-MSF* genes (*SPX-MSF1*, *SPX-MSF2*). It is also likely that *MIR827* overexpression compromises Pi transport processes across the tonoplast. Before concluding that compensatory mechanisms exist for the control of cellular Pi homeostasis in loss-of-function and gain-of-function rice plants, a better knowledge of the transport activity of rice transporters (efflux, influx activities), particularly SPX-MSF transporters, is needed.

Links between the Arabidopsis Pi starvation signalling pathway have been previously reported (Castrillo *et al.*, 2017). Here, the PHR1 transcription factor was found to negatively regulate the expression of defense-related genes under low Pi conditions. Enhanced resistance to infection by *Pseudomonas syringae* was observed in phr1

mutants (Castrillo *et al.*, 2017) . As previously mentioned, miR399 overexpression increases susceptibility to *M. oryzae* infection in rice (Campos-Soriano *et al.*, 2020).

Another finding of this study is that, miR827 accumulation was completely abolished using the CRISPR/Cas9-mediated genome editing system, thus, demonstrating the usefulness of this technology for the functional analysis of *MIR* genes in rice. Deletions were generated in the *MIR827* locus using two gRNAs targeting gene regions encompassing the miRNA precursor. These deletions would impair proper precursor processing and accumulation of mature miR827 species as revealed by the absence of mature miR827 species in leaves of CRISPR/Cas9 rice plants. It is anticipated that the use of the CRISPR/Cas9 technology will have important implications for targeted mutagenesis of *MIR* genes playing a role in disease resistance in rice plants, an aspect that still remains poorly explored.

Results here presented highlight the importance of Pi in determining disease resistance in rice. The functional characterization of gain-of-function and loss-of-function miR827 rice plants support that miR827 plays a role in controlling resistance to the blast fungus *M. oryzae*. Not only total Pi accumulation, but also alterations in the subcellular distribution of Pi might be important factors in processes controlling rice immunity. These observations further support that cross-talk Pi signaling and disease resistance occurs in plants. To better understand how rice plants defend themselves during *M. oryzae* infection, it is essential to take into account that plants can be exposed to inadequate Pi supply, thus, affecting disease resistance. In addition to Pi supply, it has long been recognized that high nitrogen fertilization enhances susceptibility to the blast fungus in rice (Ballini *et al.*, 2013). Since blast is a major threat in rice production, a better knowledge of cross-talk between Pi signaling pathways and immune responses will lay a foundation for the rational use of fertilizers and pesticides and will help in designing novel strategies to improve blast resistance in rice.

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^{31}P-HR-MAS (^{31}P-High Resolution-Magic Angle Spinning Nuclear Magnetic Resonance) experiments on rice were carried out at the NMR Facility of the Universitat Autònoma de Barcelona (UAB; https://sct.uab.cat/sermn/) which is also part of NANBIOSIS ICTS, Unit 25 (https://www.nanbiosis.es/portfolio/u25-nmr-biomedical-application-i/). We thank Miquel Cabañes, Servei de RMN, Universitat Autònoma de Barcelona, for assistance in ^{31}P-HR-MAS experiments on rice.

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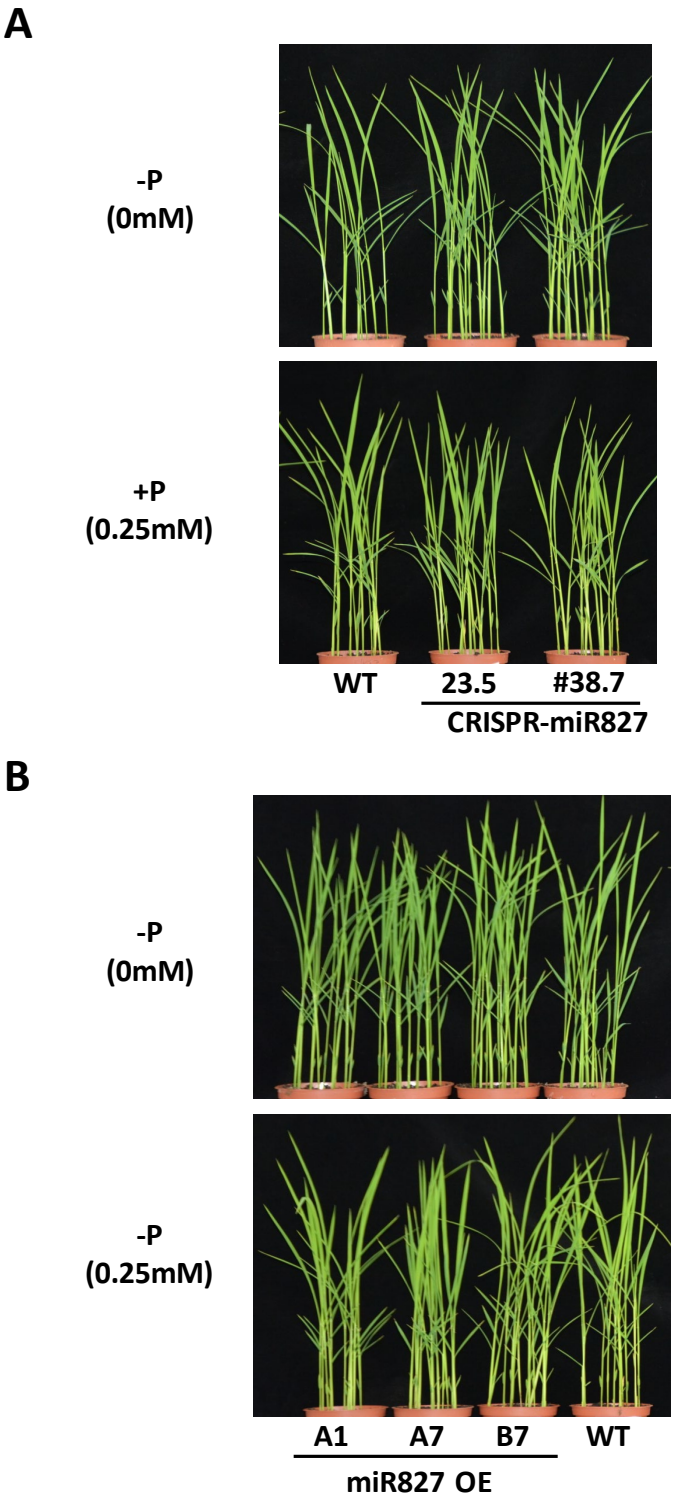
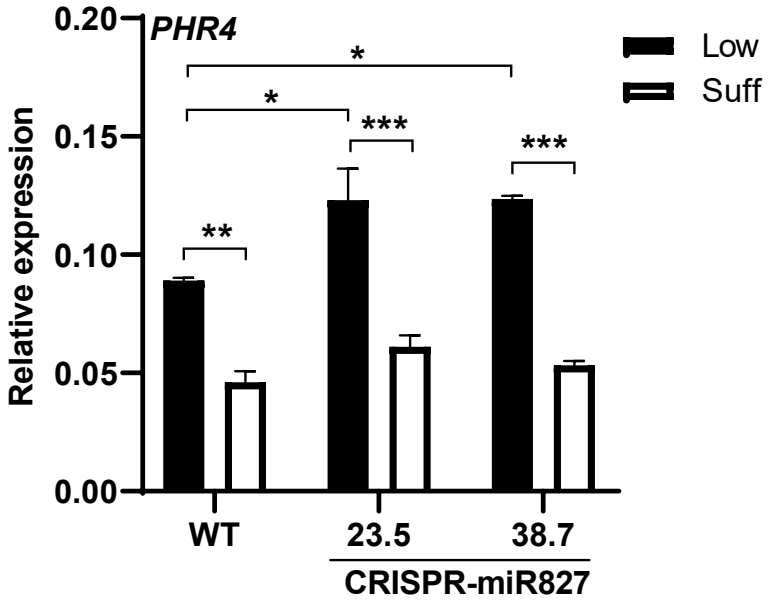
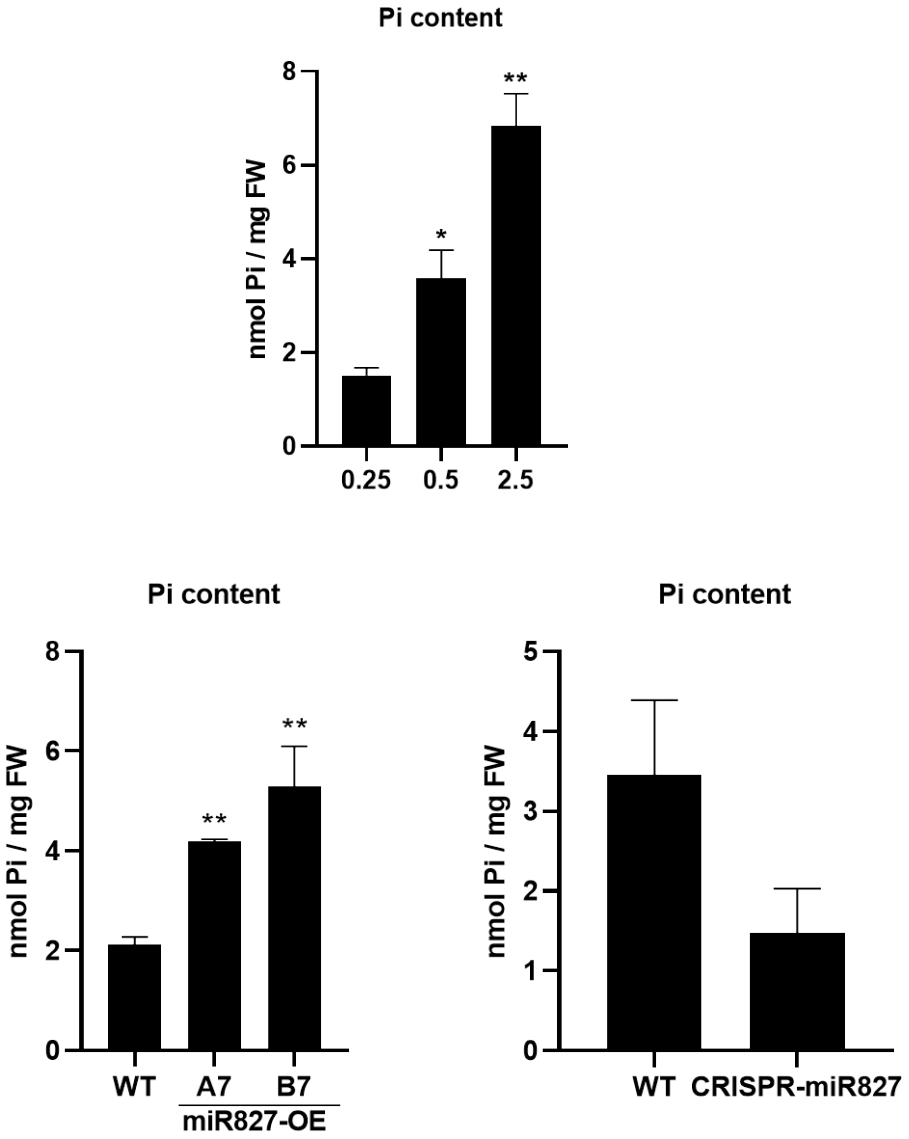
SUPPLEMENTAL MATERIAL



Supplemental Figure S1 Phenotype of CRISPR-miR827 (lines #23.5 and #38.7) and wild-type (WT) plants grown under low Pi and sufficient Pi supply conditions.



Supplemental Figure S2. *OsPHR4* expression in wild-type and CRISPR-miR827 plants grown under low or sufficient Pi conditions. Data from one representative experiment of three independent experiments are presented (each experiment consisting of a pool of 3 individual leaves) (*Two-way ANOVA* test, *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$).



Supplemental Figure S3. Quantification of total Pi in plants that have been grown *in vitro* under different Pi supply. The same plants were also subjected to ^{31}P-HRMAS analysis. Values are the mean ± SEM of four biological replicates and three plants per biological replicate (*t-test*, *$p \leq 0.05$ and **$p \leq 0.01$).

A. Total Pi content of WT plants grown under different Pi supply (0.25mM, 0.5mM and 2.5mM).

B. Total Pi content of WT and miR827-OE lines (left panel). Total Pi content of WT and CRISPR-miR827 lines (right panel).

Supplemental table S1 List of oligonucleotides

Gene name	Accession Number		Sequence (5'-3')
For expression analysis			
OsUBQ1	*Os06g0681400*	Fw	TTCCCCAATGGAGCTATGGTT
		Rv	AAACGGGACACGACCAAGG
Mo28S		Fw	TACGAGAGGAACCGCTCATTCAGATAATTA
		Rv	TCAGCAGATCGTAACGATAAAGCTACTC
Pre-miR827		Fw	GTGCATGCCTGGAGATTGGAG
		Rv	CTGATGGTCATCTAACCACGC
miR827 stem loop		Rt	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTGTTTG
		Fw	AGGCGGTTAGATGACCATCAG
SPX-MFS1	*Os04g48390*	*Fw*	GTGCCTGATGAATCTGCGTC
		Rv	CCGAGTCCTCCGGGCTA
SPX-MFS2	*Os02g45520*	Fw	CTGGCCTCAGCGCTTGGT
		Rv	GGTGATTCCCTGATTTCGTCCT
miR827_CRISPR-genot		Fw	ACGGGAGCAGAGATCGAAGAA
		Rv	CTCTGGGCTTGCTAATTGCTACT
PHR4	*Os06g49040*	Fw	GCAAGGATTTCTCAGCTGCAT
		Rv	CTGCTTCCAAATTCAGATTCTCAT

General discussion

General discussion

During the last years, advances in next-generation sequencing (NGS) technologies have had a great impact *on the characterization of* miRNA population, or miRNAome, from different plant tissues and/or developmental states, as well as in adaptation to environmental stresses. Although a substantial fraction of the miRNAome has been shown to be regulated during pathogen infection in plants, the biological function of most pathogen-regulated miRNAs remains elusive.

The regulatory networks underlying the plant response to pathogen infection are complex, and host defense responses to pathogen infection can be greatly influenced in plants that are simultaneously exposed to an abiotic stress (e.g. drought, temperature). On the other hand, although data available in the literature supports the involvement of distinct miRNAs in controlling either nutrient stress responses or immune responses, our knowledge on miRNAs playing a role in crosstalk between nutrient stress and immune signaling is still scarce.

In this PhD Thesis we sought to investigate the effect of phosphate content in resistance to pathogen infection in Arabidopsis and rice plants. The rational for conducting this research came from data previously obtained in our group on small RNA sequencing of rice tissues treated with elicitors obtained from the rice blast fungus *M. oryzae*. In that study, a large set of pathogen-regulated miRNAs potentially involved in rice immunity was identified. Among them, there were several miRNAs with a known function in nutrient homeostasis, such as miR399 and miR827 (phosphate homeostasis), miR395 (sulfur homeostasis) or miR398 (Copper homeostasis) (Campo *et al.*, 2013; Baldrich *et al.*, 2015) . Moreover, preliminary data in our group on miR399 overexpressor rice plants revealed that these plants overaccumulated Pi and were susceptible to infection by the rice blast fungus *M. oryzae*. Considering that nowadays fertilizers and pesticides are routinely used to obtain optimal yield and to protect crops from diseases caused by pathogens, we decided to investigate how Pi supply can modulate plant defense responses.

The main core of this Ph. D. Thesis deals with the need to investigate how Pi content might affect disease resistance in plants, and to identify molecular mechanisms

underlying disease resistance when plants are grown under contrasting Pi regimes. These studies have been carried out in Arabidopsis and rice. In addition to being an important crop, rice is also considered a model for functional genomics in monocotyledonous plants. As for Arabidopsis, a dicotyledonous species, this is the major model species in plant biology. In comparison, the mean generation time of rice is much longer than that of Arabidopsis, thus, research on rice requires large and expensive greenhouse facilities. Research in rice is further complicated by the fact that this plant is sensitive to photoperiod. Thus, rice is a short-day plant that flowers when grown under a photoinductive, short-day cycle. In particular *O. sativa* cv. Nipponbare, the current reference genome for *japonica* subspecies of rice, is a photoperiod-dependent rice variety. When grown under greenhouse conditions, the length of *artificial illumination also affects rice growth and flowering.* Photoperiod response differs among rice cultivars, and under continually long photoperiods, some cultivars might remain vegetative for years. On the other hand, Arabidopsis has a short life cycle and small size which allow rapid culture in relatively small spaces, and numerous tools are available for genetic and genomic studies in Arabidopsis. Although Arabidopsis has little significance for agriculture, it does have a number of traits that are advantageous for basic research and it is widely used a model to understand fundamental biological processes in plants.

The information gained in this PhD Thesis demonstrated that an increase in Pi content confers resistance to infection by fungal pathogens in Arabidopsis, but disease susceptibility in rice. These findings indicate that information gained on the model Arabidopsis cannot be always being extended to other plant species (in this case, rice). While highlighting the importance of Pi in resistance to pathogen infection in Arabidopsis and rice plants, results obtained in this PhD Thesis preclude assigning a function to Pi as a general positive or negative factor in plant immunity. Different mechanisms might operate in each particular species, and accordingly, this research should be made on a *case*-by-*case* basis in plant/pathogen interactions.

In addition to its role in regulating the Pi status, results obtained in **Chapter I** and **Chapter II** indicated that miR399 and miR827 are involved in the regulation of immune responses in Arabidopsis. The analysis of gain-of-function and loss-of-function of *mir399*

and *mir827* Arabidopsis mutants, as well as mutants for the miR399 and miR827 target genes (*PHO2* and *NLA*, respectively) provided important information on how Pi-regulated processes might contribute to disease resistance in Arabidopsis. Increased Pi levels in miR399 OE and *pho2* plants was found to promote ROS production, and up-regulation of genes involved in SA and JA signaling pathways, which is consistent with increased levels of SA and JA in these plants. As a consequence, miR399 OE and *pho2* mutants exhibited resistance to infection by fungal pathogens with a necrotrophic (*P. cucumerina*) or hemibiotrophic (*C. higginsianum*) lifestyle. Similar results were observed in Arabidopsis plants that have been grown under high Pi supply, further supporting a relationship between Pi supply and immune responses in Arabidopsis plants.

Overaccumulation of Pi and disease resistance also occurs in miR827 overexpressor and *nla* mutant Arabidopsis plants. Resistance to pathogen infection in *nla* mutant plants was accompanied by higher ROS production and callose deposition. Accumulation of SA and JA, and higher expression of SA- and JA-regulated defense genes also occurs in *nla* mutant plants. Because, plant hormones do not function independently, but synergistic or antagonistic interactions between hormone pathways are known to regulate defense gene expression, there is the possibility that differences in disease resistance between Arabidopsis and rice plants might rely on a different interplay between hormone signaling pathways, which might depend on the host and/or the type of pathogen. In Arabidopsis, the activation of the JA signaling pathway is required for resistance against necrotrophic pathogens, while biotrophic and hemibiotrophic pathogens activate mainly the SA signaling pathway. Furthermore, SA can antagonize JA signaling and vice versa.

Several studies have shown that Arabidopsis deploys a wide range of inducible defense strategies against *P. cucumerina*, including JA, SA and ET, essential to restrict the growth of the pathogen (Pastor *et al.*, 2014). Whereas ethylene and salicylic acid seems to have a negative crosstalk in the regulation of defense responses against *P. syringae* in Arabidopsis, a positive co-operation exists between SA, JA and ET hormones for disease resistance to *P. cucumerina* (Sanchez-Vallet *et al.*, 2010; Sánchez-Vallet *et al.*, 2012). Both SA and JA signalling pathways play an important role in rice basal defenses against *M. oryzae* (Yang *et al.*, 2013). Rice contains high endogenous levels of SA, being resistant

cultivars more sensitive to SA signaling (Li *et al.*, 2012), and JA signaling is known to be activated upon infection with *M. oryzae* (Meng *et al.*, 2019). Differences observed in hormone interactions suggest that positive and negative crosstalk might occur depending on the type of pathogen (Berrocal-Lobo *et al.*, 2002).

Interestingly, *nla* plants accumulated a higher level of camalexin, the major phytoalexin in Arabidopsis. Camalexin is a tryptophan-derived secondary metabolite, considered the main phytoalexin involved in biotic responses in *Arabidopsis* (Glawischnig, 2007). Many genetic approaches confirmed that camalexin plays a positive role in disease resistance, and its accumulation has been correlated with resistance to different fungi such as *B. cinerea* (Ferrari *et al.*, 2003) and *P. cucumerina* (Sanchez-Vallet *et al.*, 2010).

Together, results presented in **Chapter I** and **Chapter II** in this PhD Thesis illustrated the relevance of the Pi status of Arabidopsis plants in controlling resistance to pathogen infection. Functioning of the miR399/*PHO2* and miR827/*NLA* modules plays an important role not only in Pi homeostasis, but also in the regulation of Arabidopsis immune responses. These findings further support coordination between Pi signaling and immune signaling in Arabidopsis. Regarding the miR399 and miR827 target genes, *pho2* and *nla*, these genes might function as negative regulators of Arabidopsis immunity. In this way, the activity of miR399 and miR827 on these genes would relieve this negative regulation of immune responses.

In the literature several studies support cross-talk between Pi signaling, particularly Pi-starvation signaling, and immune signaling in Arabidopsis plants (Castrillo *et al.*, 2017; Chan *et al.*, 2021). To note, *PHR1* negatively regulates defense-related gene expression in Arabidopsis, which led the authors to propose that the plant prioritize nutrition over defense (Castrillo *et al.*, 2017). Transgenic Arabidopsis plants expressing the phytoplasma effector SAP11 were found to overaccumulate Pi in leaves and were more susceptible to infection by *P. syringae* pv. *tomato* DC3000 (Lu *et al.*, 2014) .

Studies on **Chapter III** focused on rice and miR827 function in Pi homeostasis and disease resistance. To address this issue, *MIR827* mutations were generated via the CRISPR/Cas9 technology using two sgRNAs, which allowed the creation of genomic deletions in the *MIR827* locus. Compared with wild type plants, the CRISPR/Cas9-edited miR827 plants

accumulated less Pi. Conversely, miR827 overexpression overaccumulated Pi. Overaccumulation of Pi in rice enhances susceptibility to infection by the blast fungus *M. oryzae* in rice (hemibiotroph) (Campos-Soriano *et al*., 2020, **Chapter III**), an opposite phenotype of that observed in Arabidopsis plants (**Chapter I** and **Chapter II**). In comparison with Arabidopsis, Pi content appears to regulate rice immunity in a different manner.

From what we learned from gene expression analyses in high-Pi Arabidopsis plants, resistance to pathogen infection correlated with up-regulation of defense genes (present Ph. D. Thesis). Both miR399 overexpression (and down-regulation of *PHO2*) and miR827 overexpression (and down-regulation of *NLA*) positively regulates Arabidopsis defense responses. In contrast, studies in rice indicated that an increase in Pi content by either Pi treatment or miR399 overexpression enhances susceptibility to infection by the fungal pathogen *Magnaporthe oryzae* (Campos-Soriano *et al.*, 2020). Overexpression of the phosphate transporter *OsPT8*, and subsequent increase in Pi content, was also found to enhance susceptibility to *M. oryzae* infection in rice (Dong *et al.*, 2019). The regulation of defense responses in miR827 gain-of-function (miR827 OE) and miR827 loss-of-function (CRISPR-miR827) in rice remains an open question.

Differences observed in disease phenotype between Arabidopsis and rice plants might be related to differences in the target gene of miR827 in the two plant species. In Arabidopsis miR827 targets *NLA* involved in degradation of the plasma membrane-localized PHT1 phosphate transporter, while in rice miR827 targets vacuolar phosphate transporters. This fact might determine a different contribution of miR827/*NLA* and miR827/*SPX-MSF* modules in controlling cellular homeostasis in one or another plant species, an aspect that deserves further investigation.

In plant/pathogen interactions, there is a competition between the host and the pathogen for essential nutritional resources, as the pathogen must acquire nutrient elements from host tissue, and reciprocally, the host plant might defend itself by depriving the invader of nutritional resources. Foliar pathogens, as *M. oryzae* is, rely entirely on their host for nutrient supply. In this way, certain pathogens have developed ways to access certain nutrients from the host, while plants can restrict the availability

of these nutrients for defense against pathogens. Pathogen nutrition is a dynamic process that depends on the infection cycle and lifestyle of the pathogen in the host plants. There is still much to learn about how plant nutrient supplies are utilized by phytopathogenic fungi, and how these activities are controlled in the two interacting partners.

On this basis, in this PhD Thesis we investigated alterations in the vacuolar-to-cytoplasmic Pi ratio in miR827 overexpressor and CRISPR/Cas 9-edited miR827 rice plants (**Chapter III**). In this line of research, an effort was initially made to set up a method suitable for the analysis of vacuolar and cytoplasmic Pi content in rice plants using the non-invasive ^{31}P-MRS (Magnetic Resonance Spectroscopy). As a result, ^{31}P-HR-MAS proved to be a useful technique to assess subcellular compartmentalization of Pi in rice leaves. Compared with wild type plants, the miR827 OE and CRISPR/Cas9-edited plants showed increased and decreased vacuolar Pi content, respectively. Regarding cytosolic Pi, its level was reduced in both miR827 and CRISPR/Cas9-edited miR827 lines. As a consequence, the vac/cyt Pi ratio was not altered in CRISPR/Cas9 plants but increased in miR827 OE plants. Equally, the vac/cyt Pi ratio increased when increasing Pi supply to wild type plants. It can be hypothesized that alterations in the subcellular distribution of Pi contributes to susceptibility to *M. oryzae* infection in miR827 OE plants and Pi-treated wild type plants, whereas maintenance of vacuolar-to-cytoplasmic Pi ratio is important for resistance to infection by this fungus in CRISPR-miR827 rice plants.

Clearly, further studies are needed to decipher the mechanisms by which vacuolar Pi influx and efflux transporters fine-tune cellular Pi homeostasis. A complexity in these studies relate to the fact that several vacuolar Pi transporters have been described in rice, and different results are found in the literature regarding the function of these transporters (e.g. vacuolar influx and efflux transporters). The miR827 target gene *SPX-MFS1*, has been reported to facilitate import of Pi into the vacuole, while for *SPX-MFS2* (also a target gene for miR827) a function in Pi export across the tonoplast has been proposed (Wang *et al.*, 2012; Liu *et al.*, 2016). The precise role of *OsSPX-MFS3* in vacuolar Pi transport still remains unclear. There are reports in which rice SPX-MSF3 was described to function as a vacuolar Pi efflux transporter (Wang *et al.*, 2015). However,

the analysis of *OsSPX-MSF3* overexpressor lines and loss-of-function mutants showed that *SPX-MFS3* is a vacuolar Pi influx transporter (Xu *et al.*, 2019). Additional research on mutants for miR827 target genes might help in understanding how these genes function in maintenance of intracellular Pi homeostasis.

Collectively, results obtained in this PhD Thesis together with those previously reported by our group, demonstrated that Pi excess (e.g. due to over fertilization) negatively affects blast resistance. Also in rice, nitrogen fertilization was found to increase susceptibility to infection by the rice blast fungus. Fertilization, either Pi or N fertilization should then be considered on a cost-benefit basis in rice cultivation. The indiscriminate use of fertilizers might have undesirable effects not only on the environment but also on rice protection against blast. The information gained in this Ph. D. Thesis might lay a foundation for rationally optimizing fertilizer and pesticide use in rice production. For this, a better knowledge of the molecular mechanisms underlying crosstalk between Pi signaling and immune signaling is still needed.

Conclusions

Conclusions

Chapter I

1. Phosphate content has an effect on disease resistance in Arabidopsis. An increase in Pi content caused by miR399 overexpression and loss-of-function of *PHOSPHATE 2* (*PHO2*), as well as by growing wild type plants under high Pi conditions, leads to Pi accumulation and resistance to infection by necrotrophic (*P. cucumerina*) and hemibiotrophic (*C. higginsianum*) fungal pathogens.

2. Disease resistance in high Pi Arabidopsis plants (miR399 overexpressor, *pho2* mutant, and phosphate-treated wild type plants) is accompanied by an increase in ROS production. Stronger induction of defense-related genes involved in the salicylic acid- and jasmonic acid signaling pathways occurs in miR399 overexpressor and *pho2* mutant plants, which is consistent with increased levels of salicylic acid and jasmonic acid levels. Overall, these results suggest that miR399 plays a role in maintenance of appropriate *phosphate* homeostasis, as well as in the regulation of immune responses in Arabidopsis plants.

Chapter II

3. miR827 and its target gene *NLA* (Nitrogen Limitation Adaptation) modulate Arabidopsis immune responses via regulation of Pi content. High phosphate levels caused by miR827 overexpression or loss-of-function of *NLA* confers resistance to infection by *P. cucumerina and C. higginsianum*.

4. Loss-of-function of *NLA* positively regulates immune responses in Arabidopsis by increasing ROS production and callose deposition. Up-regulation of salicylic acid- and jasmonic-acid dependent defense signaling pathways occurs in *nla* mutant plants, which is consistent with an increase in the accumulation of salicylic and jasmonic acid.

5. Expression of genes involved in the biosynthesis of the phytoalexin camalexin, and accumulation camalexin, might also explain resistance to pathogen infection in loss-of-function of *NLA*, the miR827 target gene.

6. Collectively, results presented in **Chapter I** and **Chapter II** indicated that the miR827/*NLA* and miR399/*PHO2* expression modules play an important role in modulating immune responses in Arabidopsis, further supporting cross-talk between Pi signaling and immune signaling in plants.

Chapter III

1. In rice, CRISPR/Cas9-induced loss-of-function mutations in the *MIR827* locus enhances resistance to infection by *Magnaporthe oryzae*, the causal agent of the rice blast disease. Conversely, miR827 overexpression increases blast susceptibility.

2. Compared with wild type plants, the CRISPR/Cas9-edited miR827 rice plants have reduced Pi content, while *MIR827* overexpression increases Pi levels. Based on ^{31}P-HR MAS analysis, no alterations in the vacuolar-to-cytoplasmic Pi ratio were detected in the CRISPR/Cas9-edited miR827 plants, these plants also exhibiting blast resistance. In contrast, miR827 overexpression and treatment with Pi provoke alterations in the vacuolar-to-cytoplasmic Pi ratio while enhancing blast susceptibility. Maintenance of intracellular Pi homeostasis might be an important factor in controlling resistance to infection by *M. oryzae* in rice.

   Overall, results obtained in this PhD Theses indicated that although crosstalk between phosphate signaling and immune signaling occurs in Arabidopsis and rice plants, the consequences of an alteration in phosphate content differ between Arabidopsis and rice. In rice, Pi accumulation increases susceptibility to pathogen infection. Contrary to this, increased Pi levels in Arabidopsis plants confer disease resistance.

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ANNEX

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MiR858-Mediated Regulation of Flavonoid-Specific MYB Transcription Factor Genes Controls Resistance to Pathogen Infection in Arabidopsis

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Regular Paper

MicroRNAs (miRNAs) are a class of short endogenous non-coding small RNAs that direct post-transcriptional gene silencing in eukaryotes. In plants, the expression of a large number of miRNAs has been shown to be regulated during pathogen infection. However, the functional role of the majority of these pathogen-regulated miRNAs has not been elucidated. In this work, we investigated the role of Arabidopsis miR858 in the defense response of Arabidopsis plants to infection by fungal pathogens with necrotrophic (*Plectosphaerella cucumerina*) or hemibiotrophic (*Fusarium oxysporum* and *Colletotrichum higginsianum*) lifestyles. Whereas overexpression of *MIR858* enhances susceptibility to pathogen infection, interference with miR858 activity by target mimics (*MIM858* plants) results in disease resistance. Upon pathogen challenge, stronger activation of the defense genes *PDF1.2* and *PR4* occurs in *MIM858* plants than in wild-type plants, whereas pathogen infection induced weaker activation of these genes in *MIR858* overexpressor plants. Reduced miR858 activity, and concomitant up-regulation of miR858 target genes, in *MIM858* plants, also leads to accumulation of flavonoids in Arabidopsis leaves. The antifungal activity of phenylpropanoid compounds, including flavonoids, is presented. Furthermore, pathogen infection or treatment with fungal elicitors is accompanied by a gradual decrease in *MIR858* expression in wild-type plants, suggesting that miR858 plays a role in PAMP (pathogen-associated molecular pattern)-triggered immunity. These data support that miR858 is a negative regulator of Arabidopsis immunity and provide new insights into the relevant role of miR858-mediated regulation of the phenylpropanoid biosynthetic pathway in controlling Arabidopsis immunity.

Keywords: *Arabidopsis thaliana* • *Colletotrichum higginsianum* • Defense response • *Fusarium oxysporum* • MiR858 • *Plectosphaerella cucumerina*.

Abbreviations: CAD, cinnamyl-alcohol dehydrogenase; CaMV, *Cauliflower mosaic virus*; C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumarate-CoA ligase; DPBA, diphenylboric acid 2-aminoethyester; dpi, days post-inoculation; ET, ethylene; *FOC*, *Fusarium oxysporum* f. sp. *Conglutinans*; F3H, flavonol-3-hydroxylase; F3'H, flavonol-3'-hydroxylase; FLS, flavonol synthase; GUS, β-glucoronidase; JA, jasmonic acid; miRNA, microRNA; hpi, hours post-inoculation; MYB, V-myb myeloblastosis viral oncogene homolog; OE, overexpressor; PAL, phenylalanine ammonia-lyase; PAMP, pathogen-associated molecular patterns; PTI, pathogen-triggered immunity; RT–qPCR, reverse transcription–quantitative PCR; ROS, reactive oxygen species; SA, salicylic acid; TIR1, transport inhibitor response.

Introduction

As sessile organisms, plants have evolved multiple mechanisms to perceive and efficiently respond to potential pathogens which involve extensive transcriptional reprogramming of defense gene expression. Immunity is initiated by the recognition of microbial molecular signatures, collectively named pathogen-associated molecular patterns (PAMPs), by host pattern recognition receptors (PRRs) (Jones and Dangl 2006, Boller and He 2009, Zipfel 2014). Sensing PAMPs triggers a general defense response referred to as PAMP-triggered immunity (PTI), which operates against most pathogens (Bigeard et al. 2015). Among others, PTI components include production of reactive oxygen species (ROS), reinforcement of the cell wall by deposition of lignin, activation of protein phosphorylation/dephosphorylation processes and accumulation of antimicrobial compounds (e.g. phytoalexins). The induction of a group of genes known collectively as *Pathogenesis-Related* (*PR*) genes is also a ubiquitous response of plants to pathogen infection (van Loon et al. 2006). To counteract this innate defense, pathogens can deliver virulence effector proteins into plant cells that suppress PTI (Boller and He 2009). In turn, many plants have evolved Resistance (R) proteins that directly or indirectly detect microbial effectors. This recognition triggers a rapid and effective host defense response, the so-called effector-triggered immunity (ETI), which is highly specific (isolate, race or pathovar specific) (Cui et al. 2015). Treatment with microbial elicitors triggers the same responses that are observed in infected tissues (Boller and Felix 2009). Immune responses against fungal and bacterial pathogens have been traditionally

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considered as protein-based defense mechanisms, largely independent from the RNA-based mechanisms that typically operate in antiviral defense. The phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) function as key signaling molecules in disease resistance in plants (Glazebrook 2005, Denance et al. 2013). JA and ET might act synergistically or antagonistically in regulating plant defense against pathogen infection, whereas ET/JA and SA signaling pathways often interact in an antagonistic manner.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that direct post-transcriptional gene silencing through sequence-specific cleavage or translational repression of target mRNAs (Llave et al. 2002, Brodersen et al. 2008). The crucial role of miRNAs in diverse plant developmental processes, such as leaf, flower and root development, hormone signaling and responses to abiotic stress is well documented (e.g. drought, salinity, cold, heat, oxidative stress and nutrient deficiency) (Palatnik et al. 2003, Mallory et al. 2004, Chiou et al. 2006, Chen 2009, Jeong and Green 2013). Increasing evidence also supports that miRNAs play a role in the plant response to pathogen infection (Campo et al. 2013, Staiger et al. 2013, Weiberg et al. 2014, Borges and Martienssen 2015). The first evidence for miRNAs affecting pathogen defense came from *Arabidopsis thaliana*, where treatment with the flagellin-derived elicitor peptide flg22 from *Pseudomonas syringae* causes an increase in miR393, a negative regulator of TIR1/AFB auxin receptors. The miR393-mediated repression of auxin signaling enhances resistance to bacterial pathogens (Navarro et al. 2006). However, although a substantial fraction of the miRNA transcriptome has been shown to be responsive to pathogen infection in different plant species, the exact role of most of these pathogen-regulated miRNAs in plant immunity remains elusive. Our current knowledge on distinct miRNAs involved in disease resistance comes mainly from studies in Arabidopsis plants infected with the bacterial pathogen *P. syringae*, and less is known about miRNAs mediating resistance against fungal pathogens.

On the other hand, the general phenylpropanoid pathway metabolism is known to produce an enormous array of secondary metabolites that fulfill many vital biological functions during plant development and responses to environmental cues (e.g. UV protection, defense responses against insect herbivory, flower coloring and auxin transport inhibition) (Naoumkina et al. 2010, Falcone Ferreyra et al. 2012). The phenylpropanoid pathway is required for the biosynthesis of flavonoids and monolignols, the building blocks of lignin. In Arabidopsis, distinct members of the MYB (V-myb myeloblastosis viral oncogene homolog) family of transcription factors function as transcriptional activators of genes involved in flavonoid biosynthesis, namely the *AtMYB11*, *AtMYB12* and *AtMYB111* genes (Mehrtens et al. 2005, Stracke et al. 2007, Liu et al. 2015). It is also known that miR858 targets, and cleaves, *AtMYB11*, *AtMYB12* and *AtMYB111* transcripts (Sharma et al. 2016, Fahlgren et al. 2007, Addo-Quaye et al. 2008, Dubos et al. 2010).

In this study, we investigated the potential role of miR858 in Arabidopsis disease resistance. Transgenic plants overexpressing *MIR858* were found to be more susceptible to infection by fungal pathogens whereas interference with miR858 activity by the target mimic strategy (*MIM858* lines) confers pathogen resistance. Fungal pathogens with a necrotrophic (*Plectosphaerella cucumerina*) or hemibiotrophic [*Colletotrichum higginsianum* and *Fusarium oxysporum* f. sp. *conglutinans* (*FOC*)] lifestyle were assayed in this work. Resistance to pathogen infection in *MIM858* plants is associated with a stronger induction of ET-mediated defense responses and flavonoid accumulation, but not lignification. The antifungal activity of flavonoids (e.g. naringenin and kaempferol) and the phenylpropanoid *p*-coumaric acid is presented. Overall, results presented here support that miR858 functions as a negative regulator of Arabidopsis immunity by controlling accumulation of antifungal phenylpropanoid compounds.

Results

Increased susceptibility to infection by fungal pathogens in Arabidopsis plants overexpressing *MIR858*

In *A. thaliana*, miR858 is encoded by two loci, *MIR858A* and *MIR858B*. Mature miRNAs, miR858a and miR858b, are 21 nucleotides in length and differ in the last nucleotide, at both the 5′ and the 3′ end (miR858a, 5′-UUUCGUUGUCUGUUCGACCUU-3′; miR858b, 5′-UUCGUUGUCUGUUCGACCUUG-3′). To investigate whether miR858 plays a role in Arabidopsis immunity, we overexpressed either *MIR858A* or *MIR858B* under the control of the *Cauliflower mosaic virus* (*CaMV*) *35S* promoter (hereafter *MIR858A* OE and *MIR858B* OE plants, respectively). Control Arabidopsis plants carrying the empty vector were also produced. The transgenic Arabidopsis lines overexpressing a *MIR858* gene accumulated higher levels of the corresponding pre- and mature miR858 sequences (Supplementary Fig. S1A). No phenotypic differences were observed between *MIR858* OE and control plants as determined by visual inspection (Supplementary Fig. S1B) and number of rosette leaves (Supplementary Fig. S1C).

The *MIR858* OE plants were tested for resistance to infection by the fungus *P. cucumerina*. The Arabidopsis/*P. cucumerina* pathosystem is a well-established model for studies on basal resistance to necrotrophic fungi (Llorente et al. 2005). This fungus causes sudden death and blight in different crop species (i.e. melon, soybean, snap bean, pumpkin, squash, zucchini or white luppine), and also infects *A. thaliana*. As controls, *agb1.2* (impaired in the heterotrimeric G-protein β-subunit) and *lin1* (impaired in the expression of the high affinity nitrate transporter 2.1, *NRT2.1*) mutant plants were always included in disease resistance assays against *P. cucumerina*. The *agb1.2* mutant has been shown to exhibit enhanced susceptibility to *P. cucumerina* (Llorente et al. 2005), whereas *lin1* displays resistance to this fungus (Gamir et al. 2014). Of interest, *MIR858A* and *MIR858B* OE plants displayed enhanced susceptibility to infection by *P. cucumerina* compared with control plants (wild-type and empty vector) (**Fig. 1A**). As expected, *lin1* and *agb1.2* plants showed resistance and susceptibility, respectively, to infection by

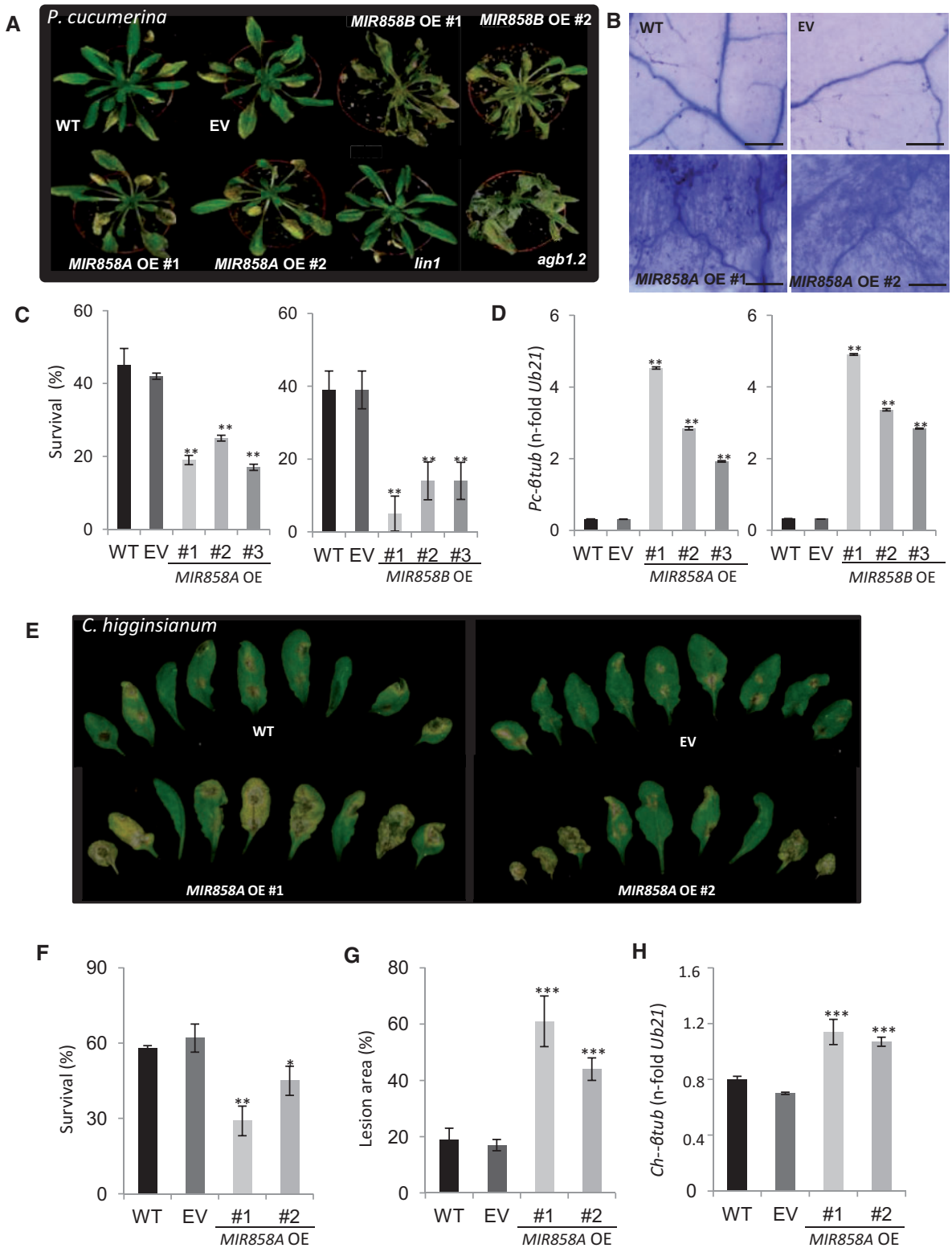

Fig. 1 Enhanced susceptibility of *MIR858* OE plants to infection by the necrotrophic fungus *P. cucumerina* (A–D) or the hemibiotrophic fungus *C. higginsianum* (E–H). Three-week-old plants were inoculated with fungal spores (1×10^5 spores ml^{-1}) or mock inoculated. Three infection experiments, each with three independent *MIR858A* and *MIR858B* OE lines (24 plants per genotype) were carried out with similar results (results obtained with lines #1 and #2 are shown). Survival (C, F) and diseased leaf area (D, G) were determined at 15 and 7 dpi, respectively. Histograms show the mean ± SD. The statistical significance was determined by ANOVA (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). (A) Phenotype of *P. cucumerina*-infected *MIR858* plants. The *agb1.2* and *lin1* mutants were used as controls (susceptibility and resistance to *P. cucumerina*, respectively). Images show the phenotype at 7 dpi with fungal spores. (B) Trypan blue staining of *P. cucumerina*-infected leaves was carried out at 72 hpi. Scale bars represent 200 μm. (C) Survival of *P. cucumerina*-inoculated *MIR858* OE plants. (D) Quantification of *P. cucumerina* DNA in

this pathogen (**Fig. 1A**). Trypan blue staining of *P. cucumerina*-inoculated leaves confirmed extensive fungal colonization in *MIR858* OE plants whereas, under the same experimental conditions, a few hyphae were observed on leaves of control plants (**Fig. 1B**). Susceptibility to fungal infection in *MIR858* OE plants was corroborated by a decrease in survival of *P. cucumerina*-infected plants (**Fig. 1C**). Moreover, qPCR analysis confirmed increased levels of *P. cucumerina* DNA in leaves of *MIR858* OE plants compared with control plants (**Fig. 1D**).

We also investigated whether *MIR858* overexpression enhances susceptibility to fungal pathogens with a hemibiotrophic lifestyle, such as *C. higginsianum*. This fungus is the causal agent of the anthracnose leaf spot disease on many cultivated forms of *Brassica* species, including *A. thaliana* (O'Connell et al. 2004). Interestingly, *MIR858* OE plants exhibited enhanced susceptibility to infection by *C. higginsianum* as revealed by visual inspection of disease symptoms, determination of plant survival and disease severity (percentage of diseased leaf area), and quantification of fungal biomass in the infected plants (**Fig. 1E-H**).

From these results, it is concluded that overexpression of *MIR858*, either *MIR858A* or *MIR858B*, increases susceptibility to infection by fungal pathogens with a necrotrophic (*P. cucumerina*) and hemibiotrophic (*C. higginsianum*) lifestyle in Arabidopsis.

Inactivation of miR858 activity by target mimics enhances resistance to infection by fungal pathogens

The use of transgenic plants designed to interfere with the activity of specific miRNAs through the target mimicry strategy (named as *MIM* plants) has proven to be a valuable resource to investigate miRNA function, including those involved in immunity (Todesco et al. 2010, Soto-Suarez et al. 2017). Target mimicry is an endogenous regulatory mechanism that plants use to regulate negatively the activity of specific miRNAs in which an endogenous long non-coding RNA (*IPS1*, *Induced by Phosphate Starvation1*) binds to miR399, but the pairing is interrupted by a mismatched loop at the expected miRNA cleavage site that abolishes the cleavage effect (Franco-Zorrilla et al. 2007). In this way, *IPS1* serves as a decoy for miR399 to interfere with the binding of this miRNA to its target transcripts, leading to miRNA degradation.

In this work, Arabidopsis *MIM858* plants were examined for pathogen resistance (for details on the production of *MIM858* plants, see Todesco et al. 2010). Although to a different extent, the accumulation of mature miR858a and miR858b sequences was significantly reduced in *MIM858* plants compared with control wild-type and transgenic empty vector plants (Supplementary Fig. S2A). The *MIM858* plants showed a normal phenotype as judged by estimation of rosette leaf number and diameter (Supplementary Fig. S2B, C). Most importantly, *MIM858* plants exhibited enhanced resistance to *P. cucumerina* infection (**Fig. 2A**; Supplementary Fig. S3). Depending on the line, 56–88% of the *MIM858* plants survived at 15 days post-inoculation (dpi), but only 21% of the wild-type and 28% of the empty vector plants survived (**Fig. 2B**). Trypan blue staining of infected leaves revealed limited fungal growth in *MIM858* and control *lin1* plants, whereas the fungus extensively proliferated in the inoculated leaves of wild-type, empty vector and *agb1.2* plants (**Fig. 2C**). qPCR analysis also revealed reduced fungal biomass in *MIM858* plants compared with control plants (**Fig. 2D**).

MIM858 plants exhibited resistance to infection by *C. higginsianum* (**Fig. 2E–H**). In agreement with visual inspection of the fungal-infected plants, *MIR858* plants that have been infected with *C. higginsianum* showed higher survival rates, reduced percentage of diseased leaf area and less fungal biomass relative to control plants (wild-type and empty vector) (**Fig. 2E–H**). The resistance phenotype of *MIM858* plants to *P. cucumerina* and *C. higginsianum* infection is consistent with the phenotype of susceptibility that is observed in plants overexpressing *MIR858*.

During the course of this work, we also examined whether interference with miR858 activity confers resistance to *FOC*, a hemibiotrophic pathogen that causes wilt disease on a broad range of plant species, including *A. thaliana* (Mauch-Mani and Slusarenko 1994, O'Connell et al. 2004). Upon pathogen challenge, chlorosis and leaf curling were evident in control plants (wild-type and empty vector), culminating in yellowing and necrosis, whereas *MIM858* lines exhibited much milder symptoms (Supplementary Fig. S4A). The *FOC*-inoculated *MIM858* plants exhibited higher survival and reduced diseased leaf area as well as less fungal biomass in their leaves compared with control plants (Supplementary Fig. S4B-D). Collectively, disease resistance assays demonstrated that *MIR858* overexpression increases susceptibility to infection by fungal pathogens, whereas interference with miR858 activity results in enhanced resistance to pathogen infection. These findings are consistent with a role for miR858 in regulating resistance to pathogen infection.

Moreover, we examined the expression of the defense-related genes *PDF1.2* and *PR4* in *MIM858* and *MIR858* OE plants. As expected, infection with *P. cucumerina* induced the expression of these genes in wild-type plants (**Fig. 3**). Note that *PDF1.2* and *PR4* expression was induced at a much higher level

Fig. 1 Continued
fungal-inoculated wild-type, empty vector and *MIR858* OE plants at 3 dpi as determined by real-time PCR using specific primers of *P. cucumerina* β-*tubulin* (values are fungal DNA levels normalized against the Arabidopsis *Ubiquitin21* gene). (E) Susceptibility of *MIR858A* OE plants to infection by *C. higginsianum*. Dissected leaves of wild-type, empty vector and *MIR858A* OE plants at 7 dpi are shown. (F, G) Survival and diseased leaf area of *C. higginsianum*-inoculated *MIR858A* OE plants. (H) Quantification of *C. higginsianum* DNA in infected wild-type, empty vector and *MIR858* OE plants at 3 dpi as determined by real-time PCR using specific primers of the *C. higginsianum ITS* region (values are fungal DNA levels normalized against the Arabidopsis *Ubiquitin21* gene).

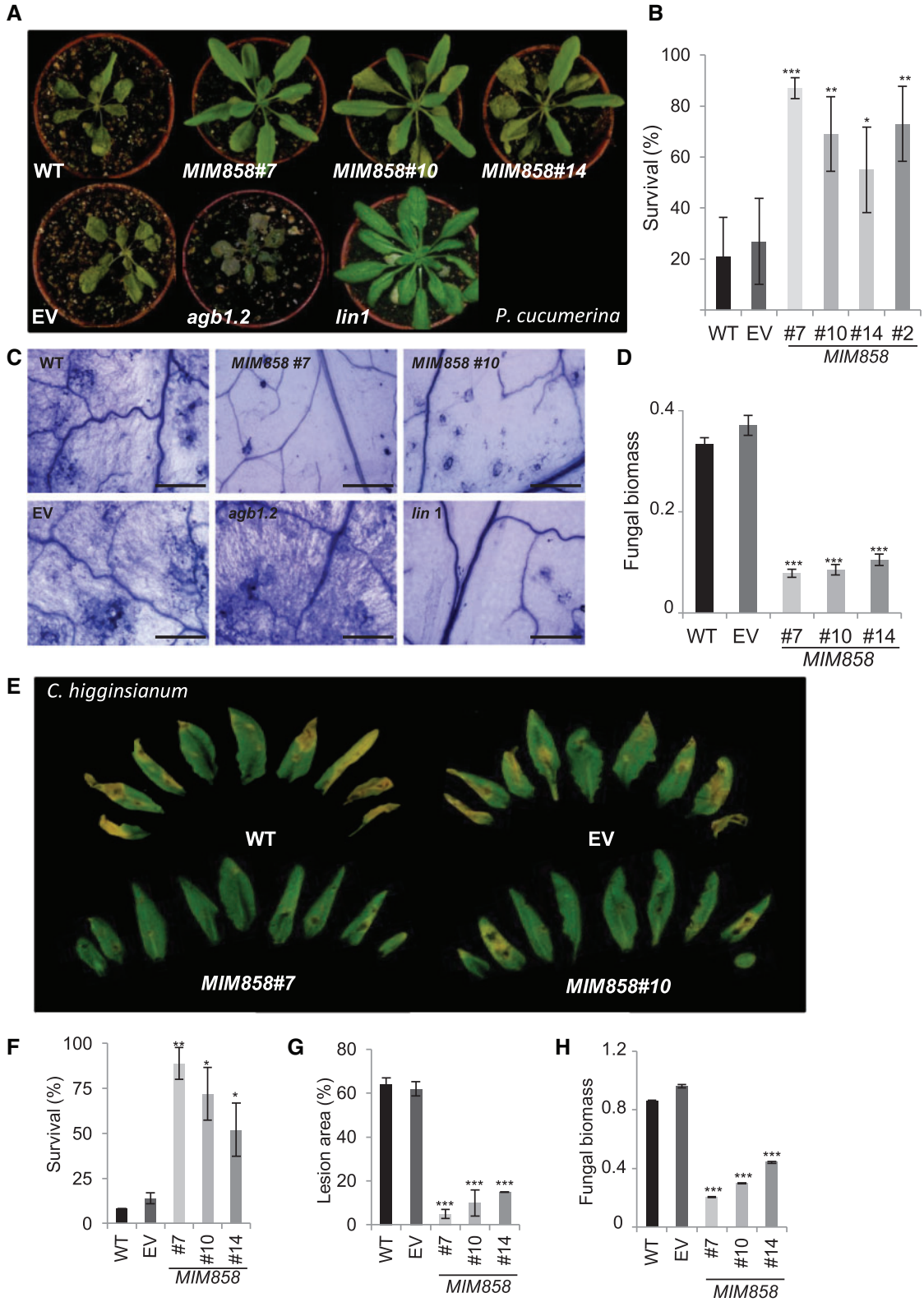

Fig. 2 Resistance of *MIM858* plants to infection by the fungal pathogens *P. cucumerina* (A–D) or *C. higginsianum* (E–H). Plants were grown for 3 weeks and then inoculated with fungal spores (1×10^6 spores ml^{-1}) or mock inoculated. Four independent *MIM858* lines were assayed (24 plants per genotype; results for lines #7, #10 and #14 are shown). Survival (B, F) and diseased leaf area (D, G) were quantified at 15 and 7 dpi, respectively. Histograms show the mean ± SD of three biological replicates (ANOVA test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). (A) Appearance of wild-type (WT, Col 0), empty vector (EV) and *MIM858* plants at 7 dpi after inoculation with *P. cucumerina* spores. The *agb1.2* and *lin1* mutants were used as controls (susceptibility and resistance to *P. cucumerina*, respectively). (B) Survival of *P. cucumerina*-inoculated *MIM858* and control plants. (C) Trypan blue staining of *P. cucumerina*-infected leaves of *MIM858* and control plants. Scale bars represent 200 µm. (D) Quantification

in fungal-infected *MIM858* plants compared with fungal-infected wild-type plants (**Fig. 3A**). In the literature there are many examples of resistance to pathogen infection in Arabidopsis with enhanced *PDF1.2* expression, including resistance to *P. cucumerina* (Berrocal-Lobo et al. 2002, Coego et al. 2005). In contrast to this, *P. cucumerina* infection induced weaker activation of *PDF1.2* and *PR4* expression in *MIR858* OE plants compared with wild-type plants (**Fig. 3B**). We did not observe important differences in pathogen responsiveness of *VSP2*, *LOX2*, *PR1a* and *NPR1* expression between *MIM858* plants and *MIR858* OE plants (Supplementary Fig. S5). As *PDF1.2* and *PR4* expression is known to be regulated by the defense-related hormone ET, the observation that *MIM858* plants respond to pathogen infection with a superinduction of *PDF1.2* and *PR4* expression, while these genes are weakly induced in *MIR858* OE plants, raises the possibility that miR858 is a negative regulator of ET-dependent signaling. Future research is needed to test whether interference with miR858 activity and/or *MIR858* overexpression has an effect on ET signaling pathways in Arabidopsis immunity.

MIR858 expression is down-regulated during fungal infection and treatment with fungal elicitors

To gather further support for the involvement of miR858 in Arabidopsis immunity, we examined the accumulation of miR858a and miR858b precursor sequences (pre-miR858a and pre-miR-858b) in wild-type plants during infection with *P. cucumerina*. A down-regulation of *MIR858* expression was observed during the entire period of infection examined here [24, 48 and 72 hours post-inoculation (hpi)] (**Fig. 4A**, left panel). The observed reduction in the accumulation of miR858 precursor transcripts in response to pathogen infection correlated well with a decrease in the accumulation of the corresponding mature miR858 sequences (**Fig. 4A**, left panel). Similarly, miR858 accumulation (precursor and mature sequences) decreased in Arabidopsis plants that have been treated with a crude preparation of elicitors obtained by autoclaving and sonicating *P. cucumerina* mycelium (**Fig. 4B**). Thus, not only pathogen infection, but also treatment with fungal elicitors results in down-regulation of *MIR858* expression, suggesting a role for miR858 in PTI.

We investigated whether the reduced level of miR858 accumulation in fungal-infected plants was the consequence of a reduced activity of the *MIR858* promoter. Towards this end, we generated transgenic plants expressing the β-*glucuronidase* (*GUS*) reporter gene under the control of the *MIR858A* promoter (*promMIR858A::GUS*). The *promMIR858A::GUS* plants were inoculated with *P. cucumerina* spores, or mock inoculated. GUS activity was monitored by histochemical (**Fig. 4C**) and quantitative fluorimetric analysis (Supplementary Fig. S6). The *MIR858A* promoter was found to be active in rosette leaves of mock-inoculated Arabidopsis plants, its activity being maintained during the entire experimental period (**Fig. 4C**, left panel). In contrast, a remarkable decrease in *MIR858A* promoter activity occurred in the *P. cucumerina*-inoculated *promMIR858A::GUS* plants that was not observed in the control *prom35SCaMV::GUS* plants (**Fig. 4C**; Supplementary Fig. S6). These results indicated that *MIR858A* expression is transcriptionally repressed during *P. cucumerina* infection.

Resistance to *P. cucumerina* infection in *MIM858* plants relies on the accumulation of phenylpropanoid compounds with antifungal activity

A miR858-guided cleavage of transcripts encoding distinct members of the large family of MYB transcription factors is well documented. The miR858 target genes are: *AtMYB11*, *AtMYB12*, *AtMYB13*, *AtMYB20* and *AtMYB111* (Fahlgren et al. 2007, Addo-Quaye et al. 2008, Sharma et al. 2016). Among them, *AtMYB11*, *AtMYB12* and *AtMYB111* are known to be involved in the biosynthesis of phytoalexins (Dubos et al. 2010). Although several other *MYB* genes are predicted to be target genes for miR858, their validation as miR858 targets is still lacking. We confirmed that *MIM858* plants accumulate higher levels of miR858-targeted transcripts than the wild type caused by mimicry-triggered miR858 degradation (Supplementary Fig. S7A). We also confirmed down-regulation of the miR858-targeted genes involved in phytoalexin biosynthesis in *MIR858* OE lines (e.g *AtMYB11*, *AtMYB12* and *AtMYB111*) (Supplementary Fig. S7B). These results indicated that expression of the flavonoid-specific *AtMYB11*, *AtMYB12* and *AtMYB111* genes is up-regulated and down-regulated in *MIM858* plants and *MIR858* OE plants, respectively. Furthermore, we observed that expression of *AtMYB11*, *AtMYB12* and *AtMYB111* is up-regulated by *P. cucumerina* infection in wild-type plants (Supplementary Fig. S7C), which is consistent with the observed down-regulation of *MIR858* expression in *P. cucumerina*-infected wild-type plants (see **Fig. 4A**). Regarding the function of the miR858 target genes *AtMYB11*, *AtMYB12* and *AtMYB111*, these transcription factors are known to function as activators of genes involved in the production of flavonols from 4-coumaroyl-CoA, namely *Chalcone synthase* (*CHS*), *Chalcone isomerase* (*CHI*), *Flavonol-3-hydroxylase* (*F3H*) and *Flavonol synthase1* (*FLS1*) (Mehrtens et al. 2005, Stracke et al. 2007, Liu et al. 2015). Here it is worth mentioning that, with the exception of flavonol synthase, genes involved in the central pathway for flavonoid biosynthesis in Arabidopsis are mostly single-copy genes. Consistent with the observed up-regulation of flavonoid-specific *MYB* transcription

Fig. 2 Continued
of *P. cucumerina* DNA in wild-type, empty vector and *MIM858* plants at 3 dpi using specific primers of *P. cucumerina* β-*tubulin* relative to the Arabidopsis *Ubiquitin21* gene. (E) Resistance of *MIM858* plants to infection by *C. higginsianum*. Dissected leaves of wild-type, empty vector and *MIM858* plants at 7 dpi are shown. (F, G) Survival and diseased leaf area of *C. higginsianum*-inoculated *MIM858* plants. (H) Quantification of *C. higginsianum* DNA in infected wild-type, empty vector and *MIM858* plants at 3 dpi as determined by real-time PCR using specific primers of the *C. higginsianum ITS* region relative to the Arabidopsis *Ubiquitin21* gene.

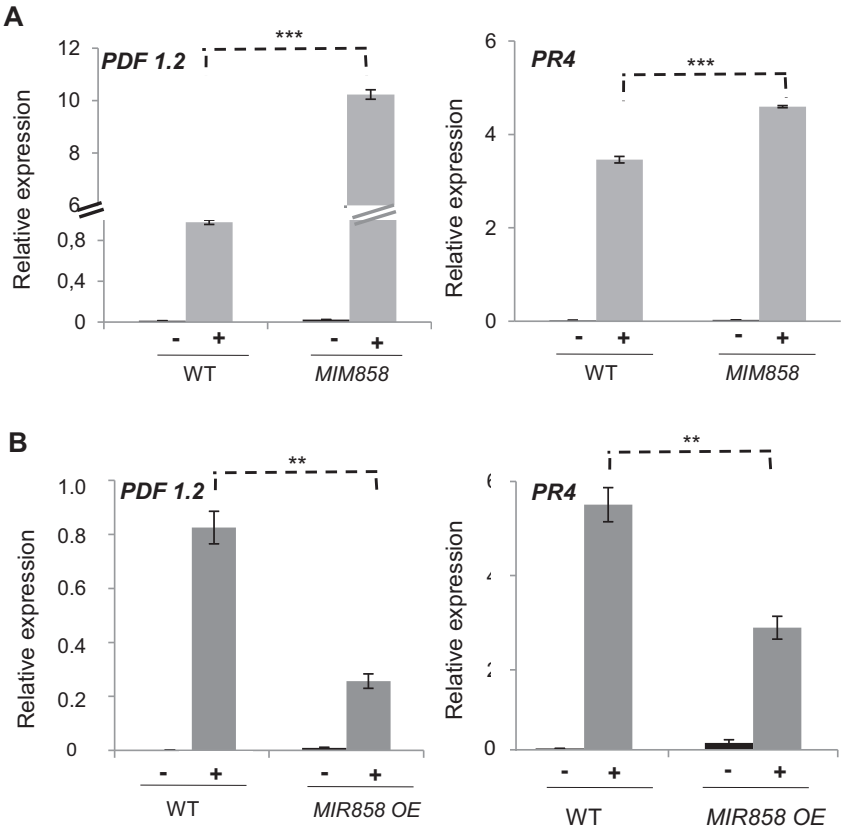

Fig. 3 Expression of the defense-related genes *PDF1.2* and *PR4* in *MIM858* (A) and *MIR858* overexpressor (B) plants in response to *P. cucumerina* infection. Three-week-old plants were inoculated (+) with *P. cucumerina* spores (1×10^6 spores ml^{-1}) or mock inoculated (−) (gray and black bars, respectively). Gene expression analyses were carried out by RT–qPCR at 3 dpi. Histograms show the mean ± SD of one out three biological replicates, each with 24 plants per genotype, with similar results. Statistical significance was determined by ANOVA (***$P \leq 0.001$).

factor genes in *MIM858* plants, metabolome analysis revealed accumulation of flavonoids in these plants (Sharma et al. 2016).

Phenylalanine serves as the precursor for the production of 4-coumaroyl-CoA, which is the precursor molecule for the flavonoid and lignin branches of the general phenylpropanoid pathway. We speculated that miR858-mediated alterations of *AtMYB11*, *AtMYB12* and *AtMYB111* expression in *MIM858* plants could have an impact on the expression of genes in the general phenylpropanoid pathway, beyond the steps in which the miR858-targeted genes function. To test this possibility, we examined the expression of genes acting upstream of the flavonoid branch in the general phenylpropanoid pathway, i.e. genes involved in the production of *p*-coumaryl-CoA from phenylalanine. They were: *PAL* (*Phenylalanine ammonia-lyase*), *C4H* (*Cinnamate-4-hydroxylase*) and *4CL* (*4-Coumarate-CoA-ligase*). Regarding *PAL* genes, up to four *PAL* genes have been identified in the Arabidopsis genome (*PAL1*, *PAL2*, *PAL3* and *PAL4*). Expression analyses revealed up-regulation of *PAL4*, *C4H* and *4CL* in *MIM858* plants, whereas their expression was down-regulated in *MIR858* OE plants (**Fig. 5A**, **B**). As for the other *PAL* genes, *PAL1* and *PAL3* were down-regulated, but *PAL2* expression was not affected, in *MIM858* plants (Supplementary Fig. S9A). *PAL1*, *PAL2* and *PAL3* expression was not significantly affected in *MIR858A* OE plants (Supplementary Fig. S9B). These findings indicate that, in addition to the miR858-targeted *MYB* genes functioning in the flavonoid branch of the phenylpropanoid pathway, genes acting upstream of the flavonoid-specific pathway (e.g. *PAL4*, *C4H* and *4CL*) are also regulated in *MIM858* and *MIR858* OE plants. Thus, interference with miR858 activity in *MIM858* plants has consequences that go beyond alterations in flavonoid-specific *MYB* genes (*AtMYB11*, *AtMYB12* and *AtMYB111*) in the general phenylpropanoid biosynthetic pathway.

Next, we investigated whether phenylpropanoid compounds, in particular flavonoids, are relevant in conferring disease resistance in *MIM858* plants. For this, we examined flavonoid accumulation in leaves of wild-type, *MIM858* and *MIR858A* OE plants that have been inoculated with *P. cucumerina* spores or mock inoculated. DPBA (diphenylboric acid 2-aminoethyl ester) staining was used to visualize flavonoid accumulation. DPBA binds to flavonoids and fluoresces in vivo, and the flavonoid–DPBA conjugates have a unique fluorescent color (e.g. yellow-green fluorescence corresponds to DPBA bound to the flavonol kaempferol) (Peer et al. 2001). Microscopic analysis of DPBA-stained leaves revealed clear differences in flavonoid accumulation between *MIM858* and wild-type plants. Under non-infection conditions, only a few tiny green fluorescence signals were distinguishable on the leaf surface of wild-type plants. In *MIM858* plants, however, larger regions showing intense green-yellow fluorescence were

consistently observed under non-infection conditions (**Fig. 6A**). The green-yellow fluorescence of flavonoid–DPBA conjugates that is observed in *MIM858* plants might well reveal kaempferol–DPBA conjugates, as previously reported (Peer et al. 2001). In favor of this possibility, a metabolomic analysis of *MIM858* plants revealed that kaempferol was the most abundant flavonoid accumulating in these plants (Sharma et al. 2016).

Importantly, whereas the flavonoid–DPBA fluorescence localized at restricted areas in leaves of mock-inoculated plants, the fluorescence exhibited a more generalized distribution in *P. cucumerina*-infected leaves (**Fig. 6A**). This generalized pattern of fluorescence was observed in fungal-infected wild-type and fungal-infected *MIM858* plants. Plants overexpressing *MIR858A* did not show accumulation of flavonoid–DPBA fluorescence, under either control or infection conditions (**Fig. 6A**).

We then hypothesized that the increased levels of flavonoids accumulating in *MIM858* plants might be responsible for the phenotype of disease resistance that is observed in these plants. In this work, we investigated the possible antifungal activity of the flavonoids naringenin and kaempferol, as well as the phenylpropanoid *p*-coumaric acid. The two flavonoids (kaempferol and naringenin) were found to be effective for inhibition of *P. cucumerina* growth, the latter having a greater antifungal activity (**Fig. 6B**). The phenylpropanoid *p*-coumaric acid was, however, more effective for inhibition of *P. cucumerina* growth than each one of the flavonoids (naringenin and kaempferol) (**Fig. 6B**). After 48 h of incubation, a concentration of 1 mM *p*-coumaric acid results in 55% inhibition of *P. cucumerina* growth (45% growth of control cultures). Increasing the concentration of *p*-coumaric acid did not significantly increase the antifungal potency of this compound. Equally, when increasing the concentration of naringenin above 2 mM (or kaempherol above 4 mM), their antifungal activity did not increase further. These findings indicate that the potency of these compounds against *P. cucumerina* might have reached maximum values under the experimental conditions assayed here. Finally, microscopic observations of fungal cultures revealed alterations in the morphology of hyphae in cultures that have been grown in the presence of either compound. Hyphae with constricted regions were frequently observed in treated cultures compared with the control cultures (Supplementary Fig. S9). These findings suggest that accumulation of phenylpropanoid compounds exhibiting antifungal activity, such as kaempferol, naringenin and *p*-coumaric acid, might be responsible, at least in part, for the disease resistance phenotype that is observed in *MIM858* plants.

Pathogen resistance in *MIM858* plants does not requires lignification

It is generally assumed that lignification plays a role in resistance to pathogen infection (Miedes et al. 2014). Lignin is deposited in the secondary cell wall, thus providing a physical barrier against pathogen invasion. However, the observation that *MIM858* plants had reduced lignification in vascular and interfascicular tissues (Sharma et al. 2016) prompted us to investigate whether resistance to *P. cucumerina* infection in *MIM858* plants depends on lignification.

Lignin accumulation was examined in mock-inoculated and *P. cucumerina*-inoculated wild-type, *MIM858* and *MIR858A* OE plants using the whole-mount phloroglucinol staining method. In the absence of pathogen infection, lignin was detected in wild-type, but not in *MIM858* plants (**Fig. 7**, upper panels). Also, the expression of the lignin-specific *CAD* (cinnamyl alcohol dehydrogenase) genes (*CAD5* and *CAD6*) involved in the synthesis of the immediate precursors of lignin was found to be down-regulated in *MIM858* plants compared with wild-type plants (Supplementary Fig. S10). Lignin accumulation greatly increased in response to fungal infection in wild-type plants, but not in *MIM858* plants (**Fig. 7**, lower panels). Finally, lignin accumulation in MIR858A plants was confirmed **(Fig. 7**, right panels).

From these results, it is concluded that lignification, most probably, does not contribute to pathogen resistance in *MIM858* plants. Down-regulation of miR858 and concomitant up-regulation of miR858 target genes appears to re-direct the metabolic flux towards the production of phenylpropanoid compounds, some of them exhibiting antifungal activity, away from lignin biosynthesis.

Discussion

In this study we provide evidence for the involvement of miR858 in Arabidopsis immunity. We show that overexpression of miR858 renders Arabidopsis plants more susceptible to pathogen infection, whereas inhibition of miR858 activity by target mimics results in enhanced resistance to infection by necrotrophic (*P. cucumerina*) and hemibiotrophic (*F. oxysporum* f. sp. *conglutinans* and *C. higginsianum*) pathogens. These findings suggest that miR858 functions as a negative regulator of disease resistance in Arabidopsis. In wild-type plants, *MIR858* expression is down-regulated not only during pathogen infection, but also in response to treatment with fungal elicitors, indicating that miR858 is a component of PTI. The increased resistance to fungal infection that is observed in *MIM858* plants is associated with a stronger induction of *PDF1.2* and *PR4* expression upon pathogen challenge. Enhanced disease susceptibility in *MIR858* OE plants is also consistent with a lower expression of these defense-related genes during pathogen infection.

Interestingly, blocking miR858 activity by target mimics results in up-regulation of the flavonoid-specific target genes *AtMYB11*, *AtMYB12* and *AtMYB111*, as well as genes upstream of the flavonoid branch in the phenylpropanoid pathway (e.g. *PAL4*, *C4H* and *4CL*). Thus, *PAL4*, *C4H*, *4CL* and miR858-regulated *MYB* genes might be regulated in a co-ordinated manner in order to prioritize flavonoid production in *MIM858* plants. At present, however, it is not possible to determine whether alterations in the expression of phenylpropanoid genes in *MIM858* plants are due to a feed-back control by metabolite levels, or to protein–protein interactions with other regulatory proteins in transcriptional complexes controlling flavonoid biosynthesis. In

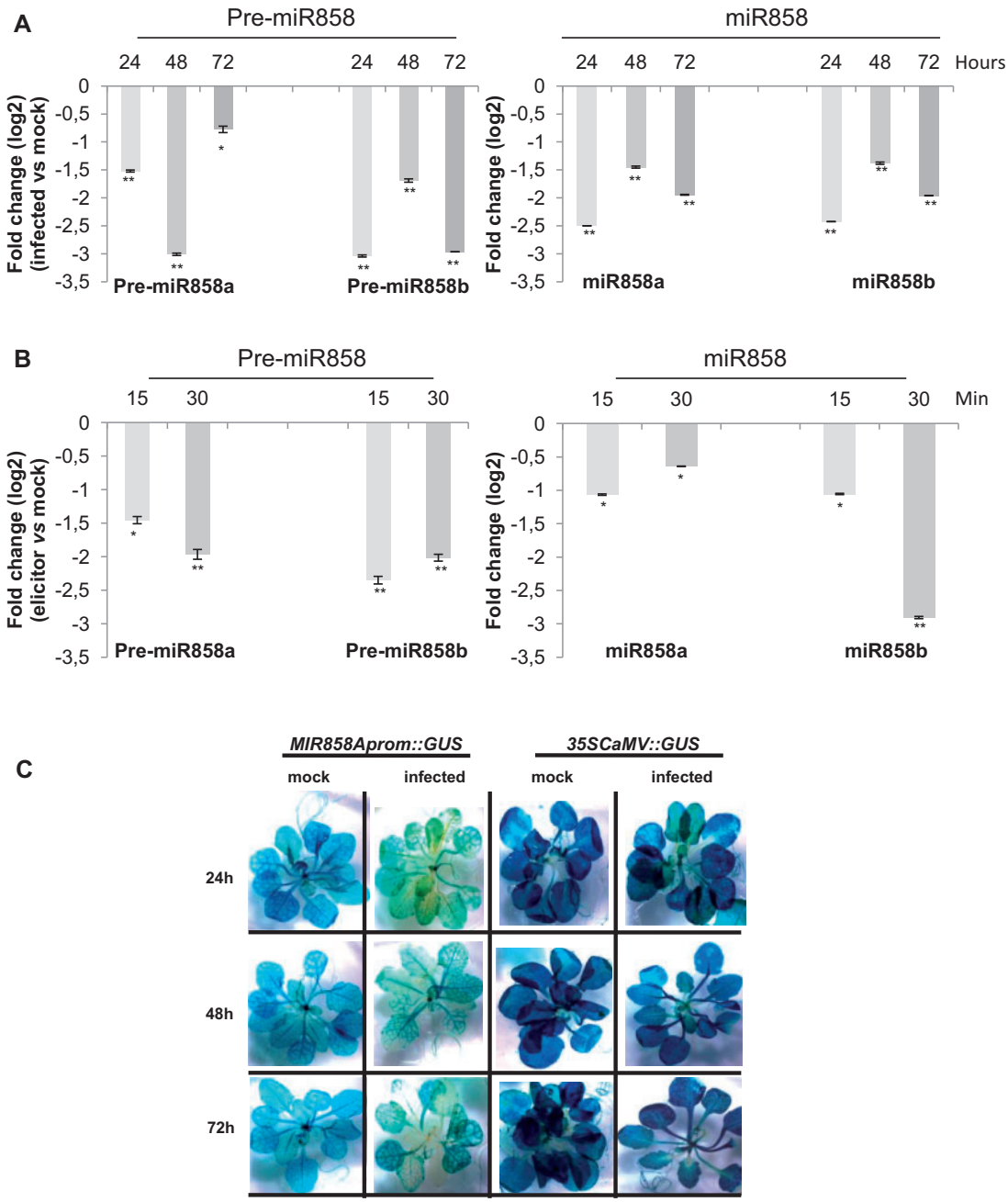

Fig. 4 Transcriptional regulation of *MIR858* induced by *P. cucumerina* in wild-type Arabidopsis plants. (A and B) Differential accumulation of pre-miR858 (left panel) and mature miR858 (right panel) in wild-type Arabidopsis (Col-0) plants treated with *P. cucumerina* spores (A) or elicitors (B) at the indicated times, as determined by RT–qPCR (pre-miR858) and stem–loop RT–qPCR (miR858) analysis. Values represent the mean fold change of pre-miR858 or miR858 in infected (A) or elicitor-treated (B) vs. mock-treated samples based in three biological replicates per time point and log-scaled. Asterisks denote statistical differences in treated vs. non-treated plants at each time point (ANOVA, $*P \leq 0.05$, $**P \leq 0.01$; $n = 3$). (C) Histochemical analysis of GUS activity in *MIR858prom::GUS* plants that have been mock inoculated or inoculated with *P. cucumerina* spores (24, 48 and 72 hpi). As control, *prom35SCaMV::GUS* plants were used.

this respect, enzymes involved in flavonoid biosynthesis have been proposed to form protein complexes, or metaboloms, to establish efficient metabolic flux of flavonoid biosynthesis (Waki et al. 2016).

Visualization of flavonoids in Arabidopsis leaves using DPBA staining revealed changes in the pattern of flavonoid accumulation between the wild type and plants with altered expression of *MIR858*. In particular, *MIM858* plants accumulated higher levels of flavonoids compared with wild-type plants, whereas flavonoids were barely detected in miR858 overexpressor plants. Furthermore, *P. cucumerina* infection induced flavonoid accumulation, the flavonoids showing a more widespread, generalized distribution in *P. cucumerina*-infected leaves than in mock-inoculated leaves. Given that flavonoids have been reported to be capable of long-distance movement in Arabidopsis (Buer et al. 2007), a widespread distribution of

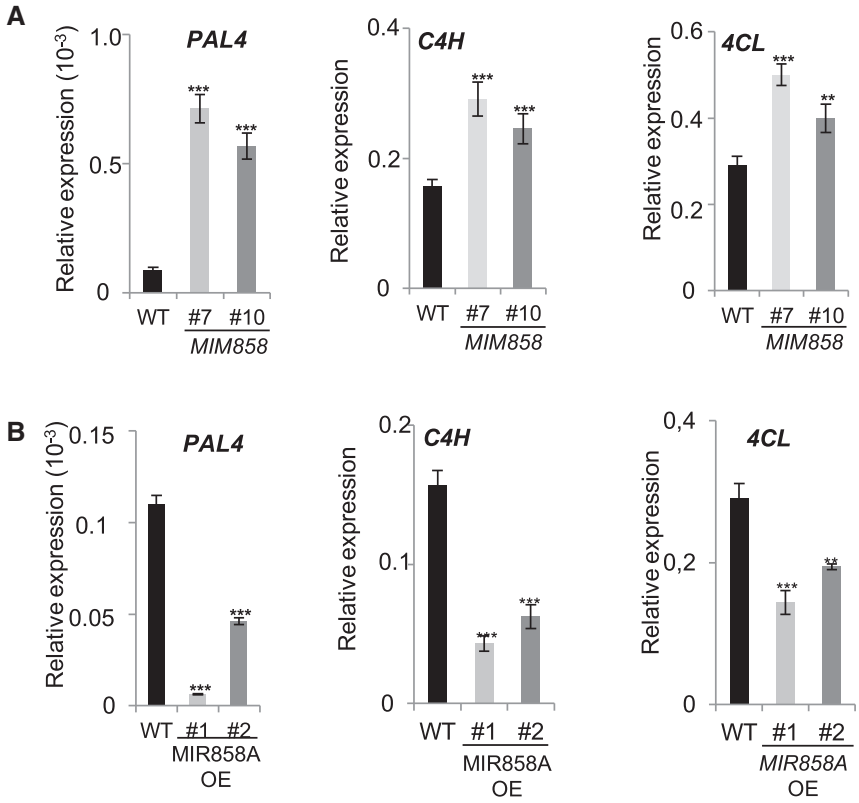

Fig. 5 Expression of genes involved in the early steps of the general phenylpropanoid biosynthesis pathway (*C4H*, *4CL* and *PAL4*) in *MIM858* (A) and *MIR858A* overexpressor (B) plants. RT–qPCR analysis was carried out using the β-*tubulin2* gene as the internal control. Histograms show the mean ± SD of two biological replicates, each with 24 plants per genotype. Statistical significance was determined by ANOVA (**$P \leq 0.01$, ***$P \leq 0.001$). C4H, cinnamate-4-hydroxylase; PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate-CoA ligase.

flavonoids in *P. cucumerina*-infected leaves points to a possible function for flavonoids as signaling molecules as part of the host defense response to pathogen infection. In other studies, flavonoids were proposed to function as signal molecules in auxin transport, or during symbiotic nitrogen fixation and mycorrhizal associations (Falcone Ferreyra et al. 2012).

Our data show that naringenin and kampherol, as well as *p*-coumaric acid, exhibit antifungal activity against *P. cucumerina*. Indeed, the antifungal potency of *p*-coumaric acid was higher than that of the two flavonoids assayed in this work. Knowing that these compounds accumulate in leaves of *MIM858* plants (Sharma et al. 2016, this study), it is likely that their accumulation contributes to antifungal resistance. The in vitro antifungal activity of the individual phenylpropanoid compounds assayed in this work (*p*-coumaric acid, naringenin and kaempferol) is, however, weaker than that of known plant antimicrobial peptides, such as lipid transfer proteins or thionins (Molina et al. 1993a, Molina et al. 1993b). Resistance to infection in *MIM858* plants might well rely on the simultaneous action and/or combined effect of antifungal activities of phenylpropanoids, including flavonoids, rather than on the activity of individual compounds. Along with this, the expression of genes involved in flavonoid biosynthesis is induced in the interaction of plants with different pathogens, and certain flavonoids (or flavonoid derivatives) isolated from plant tissues exhibited in vitro antimicrobial activity (Dai et al. 1996, Galeotti et al. 2008, Bollina et al. 2010). Concerning the mechanisms by which flavonoids exert their antifungal activity, it has been proposed that they function as ROS scavengers and chelators of metals that might generate ROS via the Fenton reaction. Potentially, flavonoids might act as antioxidant molecules in protecting the plant cell from oxidative stress induced by environmental stress (Falcone Ferreyra et al. 2012). However, the relevance of the antioxidant properties for flavonoid in the plant response to pathogen infection is still a topic of debate.

Lignification has been associated with resistance to pathogen infection in different plant species. We show that sequestration of miR858 by target mimics in *MIM858* plants leads to a reduction in lignin accumulation and down-regulation of genes encoding the specific and last step enzyme for production of monolignols (CAD5 and CAD6). Most importantly, no accumulation of lignin was observed in *P. cucumerina*-infected *MIM858* plants, supporting that resistance to fungal infection in these plants does not require a lignification response. These results also indicated that, at some level, miR858-guided regulation of flavonoid-specific *MYB* genes is involved in the cross-talk between the two phenylpropanoid branches for the production of flavonols or monolignols. Interference with miR858 activity would then re-direct the phenylpropanoid pathway towards the production of antifungal compounds, including flavonoids, at the cost of lignin synthesis. In previous studies, it was reported that flavonoids accumulate in cell walls

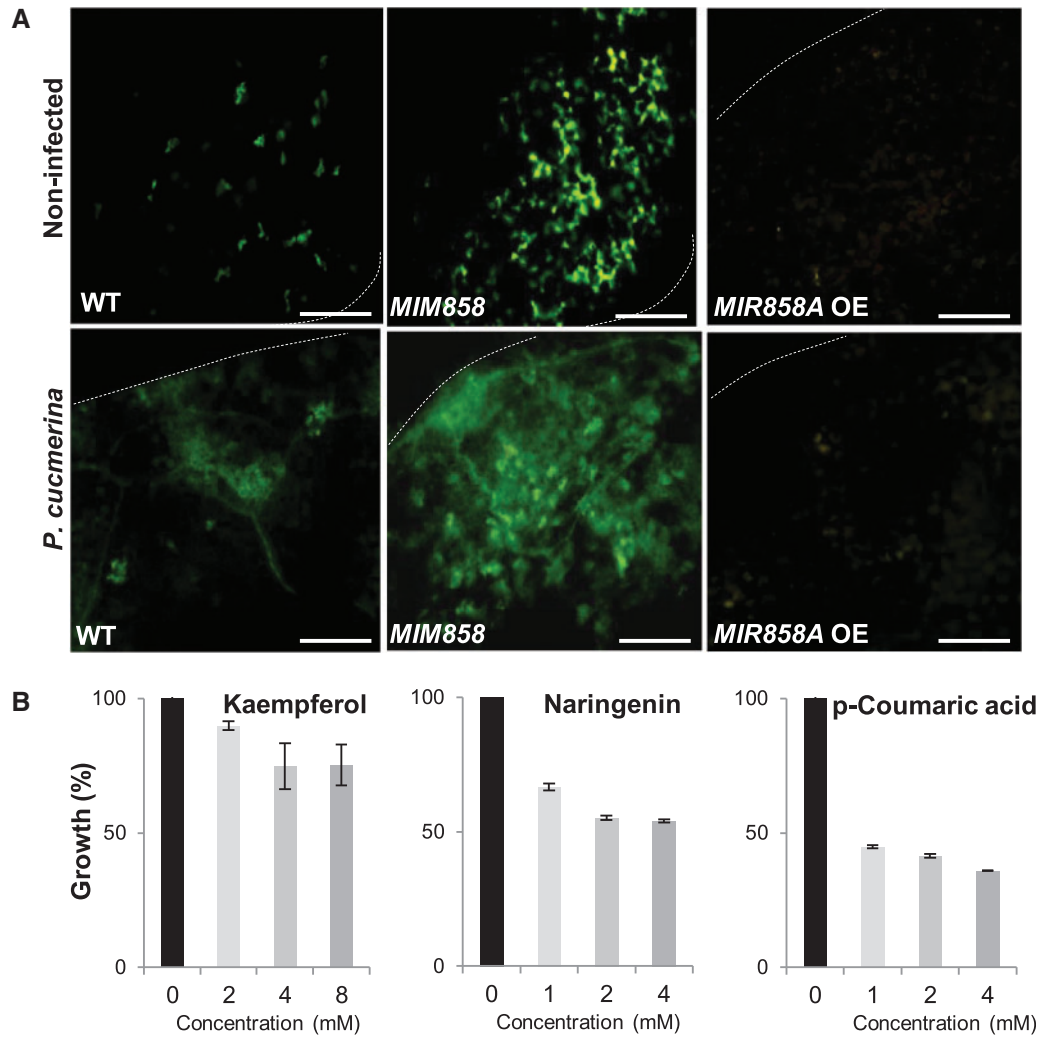

Fig. 6 In situ flavonoid detection and antifungal activity of phenylpropanoid compounds in wild-type, *MIM858* and *MIR858* OE plants. (A) Flavonoid accumulation was visualized by DPBA staining of rosette leaves. Two-week-old plants were inoculated with *P. cucumerina* spores (1×10^5 spores ml^{-1}) or mock inoculated, and subjected to DPBA staining at 72 hpi. (B) In vitro antifungal activity of the flavonoids naringenin and kaempferol, and the phenylpropanoid *p*-coumaric acid against *P. cucumerina*. Fungal cultures were grown for 48 h in PDB (potato dextrose broth) in the presence of increasing concentrations of the corresponding metabolite. Fungal growth is expressed as a percentage of the growth of control cultures (100% growth represents fungal growth in control cultures). Two repeats of each bioassay were performed for each of two different preparations of spore suspensions.

during pathogen infection (Dai et al. 1996). It is then tempting to hypothesize that a reduced lignification in *MIM858* plants may facilitate the incorporation of flavonoids in host cell walls. Further studies are, however, needed to clarify this aspect.

Under our experimental conditions, *MIR858* OE plants and *MIM858* plants grew and developed normally in the absence of pathogen infection. Differences in plant growth were, however, described in *MIR858* OE or *MIM858* plants compared with wild-type plants (Sharma et al. 2016). A possible explanation for the different growth responses in *MIR858* OE and *MIM858* plants might be the photoperiod condition used to grow the plants. In our work, plants were always grown under neutral day condition (12 h light/12 h dark photoperiod), whereas Sharma et al. (2016) grew plants under a long-day photoperiod (16 h light/8 h dark photoperiod). A photoperiod-dependent regulation of *MIR858* expression was also described (Sharma et al. 2016). Further studies are needed to establish whether links between light regulation of *MIR858* expression and growth performance exist.

Collectivelly, the results presented here demonstrate that alterations in *MIR858* expression have important consequences in disease resistance, and that Arabidopsis plants adjust their general phenylpropanoid metabolism in order to prioritize the production of phenylpropanoid compounds having antifungal activities as an effective immune response. The fact that a single miRNA, such as miR858, can regulate the expression of multiple genes involved in a specific pathway, such as the phenylpropanoid pathway, would ensure proper production of antifungal compounds as part of the plant defense response which, in turn, would increase the plant's ability to cope with pathogen infection. From a more practical point of view, this course of study can provide new ways to develop strategies to increase

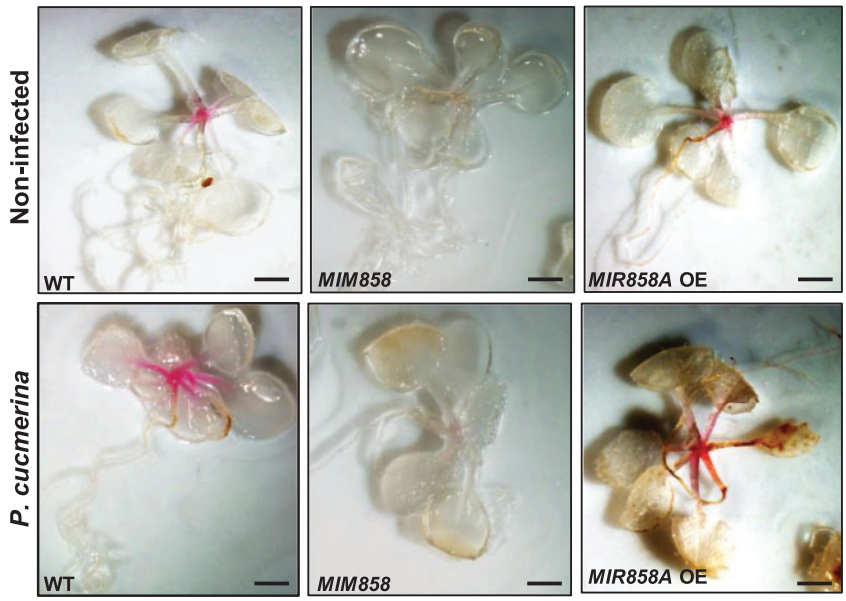

Fig. 7 Lignin accumulation in wild-type, *MIM858* and *MIR858* OE plants was determined by whole-mount phloroglucinol staining. Pictures were taken at 72 hpi with an Olympus DP71 camera. Scale bars represent 1 mm.

disease resistance in plants through inhibition of miR858 activity. In other studies, miR828 and miR858 were reported to regulate the expression of *MYB2* gene homologs that function in Arabidopsis trichome formation and cotton fiber development, these particular miRNAs also being regulated during adaptation to high temperature in cotton (Guan et al. 2014). MiR858 was also reported to mediate tolerance to drought stress in the desert plant *Ammopiptanthus mongolicus* (Gao et al. 2016). Very recently, Piya et al. (2017) described that miR858 post-transcriptionally regulates *MYB83* during cyst nematode parasitism, a process in which miR858 and *MYB83* expression appear to be connected through a feed-back circuit. Together, these observations point to a functional role for miR858 in different developmental processes and metabolism pathways, as well as during adaptation to biotic and abiotic stresses. Whether miR858 is a common component of plant adaptive responses to different types of environmental stresses needs to be further investigated. The information provided in this work extends our knowledge on miRNAs involved in plant immunity while laying the foundation for future research to uncover links between phenylpropanoid metabolism and plant immunity.

Materials and Methods

Plant and fungal materials

Arabidopsis thaliana (ecotype Columbia-0) plants were grown under a 12 h light/12 h dark photoperiod and 60% relative humidity at a temperature of 22 ± 2 °C. For in vitro assays, seeds were grown for 14 d on Murashige and Skoog (MS) medium containing 0.8% agar and vitamins. The Arabidopsis mutants *agb1.2* (Llorente et al. 2005) and *lin1* (Gamir et al. 2014) were grown as described above.

Fungi were grown at 28 °C on PDA (potato dextrose agar) supplemented with chloramphenicol (34 mg ml^{-1}). Spores were collected adding sterile water and adjusted to the desired concentration using a Neubauer counting chamber.

Generation of transgenic Arabidopsis plants

For *MIR858* overexpression, the DNA fragment containing the precursor sequence for each miR858 species was PCR amplified from genomic DNA using gene-specific primers (503 and 428 bp DNA fragments for the miR858a and miR858b precursor, respectively). Primers are listed in Supplementary Table S1. Precursor DNA sequences were cloned into the pCAMBIA1300 binary vector under the control of the CaMV35S promoter.

To obtain the *MIR858Apromoter:GUS* construct, the DNA sequence of the *MIR858A* promoter region was extracted from the NCBI (http://www.ncbi.nlm.nih.gov). The transcription start site was identified by using the transcription start site identification program for plants (http://linux1.softberry.com/). The DNA sequence covering 2 kb upstream of the transcription start site of *MIR858A* was PCR amplified from genomic DNA, and cloned into the pCAMBIA1391z plant binary vector. All PCR products were verified by sequencing. The plant expression vectors were transferred to the *Agrobacterium tumefaciens* strain GV3101. Arabidopsis (Col-0) plants were transformed using the floral dip method. Transgenic lines harboring the empty vector (pCAMBIA1300 or pCAMBIA1391z) were also obtained and used as controls.

Fungal infection and elicitor treatment

Three-week-old Arabidopsis plants were spray-inoculated with a spore suspension of *P. cucumerina* at the appropriate concentration. In each case, at least three independent transgenic lines for each genotype were assayed (*MIR858A* OE or *MIR858B* OE, and *MIM858* lines). As controls, wild-type and empty vector plants were assayed. The *agb1.2* (susceptible) and *lin1* (resistant) mutants were included in infection experiments with *P. cucumerina*. Infection assays with *FOC* were performed by applying the spore suspension to the soil near the base of the plant (200 µl, 1×10^6 spores ml^{-1}). Inoculations with *C. higginsianum* were carried out by placing two drops of the spore suspension on each leaf. Infected plants, as well as mock-inoculated plants were maintained under high humidity for the required period of time. The progress of disease symptoms was followed with time. Elicitor treatment was performed by spraying 3-week-old plants with an elicitor extract obtained from *P. cucumerina* (300 µg ml^{-1}) as described (Casacuberta et al. 1992). Three independent experiments (infection with fungal spores or treatment with fungal elicitors) were performed with at least 24 plants per genotype in each experiment. Statistically significant differences among genotypes were determined by one-way analysis of variance (ANOVA) test.

Lesion areas were quantified with the ASSESS v2.0 software on four inoculated leaves per plant (24 plants per genotype). Quantification of fungal DNA

on infected leaves was carried out by real-time PCR using specific primers for the corresponding fungus and the Arabidopsis *UBIQUITIN21* (*At5g25760*) gene as an internal control (Soto-Suarez et al. 2017). PCR primers are listed in Supplementary Table S1.

For trypan blue staining, leaves were fixed by vacuum infiltration for 1 h in ethanol : formaldehyde : acetic acid (80 : 3.5 : 5 by vol.), stained with lactophenol blue solution for 4 h and washed with 70% ethanol for 5 min. Leaves were placed in glass slides with glycerol and observed using a Zeiss Axiophot microscope.

Expression analysis by RT–qPCR and stem–loop RT–qPCR

Total RNA was extracted from rosette leaves using the TRIzol Reagent (Invitrogen). Reverse transcription reactions were performed using DNase-treated total RNA (1 μg), reverse transcriptase (Applied Biosystems) and oligo$(dT)_{18}$ (Sigma, Aldrich). RT–qPCR (reverse transcription–quantitative PCR) was performed in optical 96-well plates in a Light Cycler 480 (Roche) using SYBR® Green. Primers were designed using Primer3 software (http://www.ncbi.nlm.nih.gov). The average cycle threshold (Ct) values were obtained by PCR from three independent biological replicates and normalized to the average Ct values for the *beta-tubulin2* gene from the same RNA preparations, yielding the ΔCt value or normalized expression (relative expression). The $2^{-\Delta\Delta Ct}$ method was used to analyze relative changes in gene expression or fold change (infected/elicitor-treated vs. mock-inoculated) and visualized by log2 transformation. Primers used for RT–qPCR and stem–loop RT–PCR are listed in Supplementary Table S1. ANOVA tests were used to evaluate differences in gene expression.

Analysis of GUS activity

Histochemical staining of GUS enzyme activity was performed according to Jefferson et al. (1987). Briefly, leaves were fixed by vacuum infiltration for 1 h in ethanol : formaldehyde : acetic acid (80 : 3.5 : 5 by vol.), stained with lactophenol blue solution for 4 h and washed with 70% ethanol (5 min). Leaves were placed on glass slides with glycerol and observed using a Aixophot DP70 microscope. Quantitative GUS activity assay was carried out using the fluorimetric substrate 4-methylumbelliferyl-β-d-glucoronide (MUG) at a final concentration of 1 mM.

Determination of lignin content

Lignin accumulation was determined by whole-mount fluoroglucinol staining. For this, the Arabidopsis seedlings were fixed on 70% ethanol for 24 h, stained with phloroglucinol (0.012 mg ml^{-1} ethanol : HCl 50 : 50 v/v) for 2 min and washed with water (5 min). Leaves were placed on glass slides with glycerol and observed on an Olympus DP71 microscope.

In vivo staining of flavonoids

Flavonoids were visualized in vivo by the fluorescence of flavonoid-conjugated DPBA following the protocol described by Watkins et al. (2014). Briefly, the leaves were excised and submerged in an aqueous solution containing 0.01% (v/v) Triton X-100 and 2.52 mg ml^{-1} DPBA for 2.5 h. Leaves were then washed in deionized water for 1 min. Fluorescence was recorded on an AixoPhotDP70 microscope with excitation at 488 nm. The DPBA fluorescence emission was collected between 520 and 600 nm.

In vitro antifungal assays

The in vitro antifungal activity of naringenin, kaempferol and *p*-coumaric acid was determined by measuring the absorbance of fungal cultures at 595 nm in 96-well microtiter plates (Cavallarin et al. 1998). In microtiter plates, 150 μl of potato dextrose broth (PDB) medium containing chloramphenicol (0.03 μg μl^{-1}) were mixed with 50 μl of *P. cucumerina* spores (106 spores ml^{-1}). Spores were allowed to germinate for 6 h. The secondary metabolite was then added to the desired final concentration. The microtiter plates were incubated at 25°C for 48 h and the absorbance was read (OD 595 nm). Fungal growth was also checked microscopically to confirm the spectrophotometric data. As a control, the antifungal agent nystatin was used (0.1 mg ml^{-1}).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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