Universitat Autònoma de Barcelona

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PhD thesis

Function of microRNAs in plant innate immunity

Dissertation presented by Rosany del Carmen Camargo Ramírez for the degree of Doctor in Biology and Plant Biothecnology by Universitat Autònoma de Barcelona.

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"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale".

Marie Curie

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Ya que he finalizado esta etapa en mi vida, la manera de agradecer a todas las personas que han estado presentes de una forma u otra a lo largo de este viaje, es dedicando un trozo de este trabajo a cada uno de ellos.

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Abbreviations

ABA	Abscisic acid
amiRNA	Artificial miRNA
bHLH	*Basic helix-loop-helix*
bp	Base pairs
BR	Brassinosteroid
bZIP	Basic-region leucine zipper
CAD	Cinnamyl alcohol dehydrogenase
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CDPK	Calcium-dependent protein kinase
cv	Cultivar
DCL	DICER-LIKE
DNA	Deoxyribonucleic acid
Dpi	Days post-infection
ERF	Ethylene-responsive factors
ET	Ethylene
ETI	Effector-triggered immunity
EV	Empty vector
TF	Transcription factor
GA	Gibberellic acid
GUS	β-glucuronidase
Hpi	Hours post-infection
HR	Hypersensitive response
PSI	Phosphate Starvation Induced
IRRI	International Rice Research Institute
JA	Jasmonic acid and jasmonates
LB	Left border
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
Mb	Megabases

mRNA	Messenger RNA
miRNA	microRNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NB-LRR	Nucleotide-binding/leucine-rich repeat
Nos	Nopaline synthase
NO	Nitric oxide
NPR1	Non-expressor of pathogenesis-Related1
O2-	Ion superoxide
O2-2	Ion peroxide
OH-	Hydroxyl radical
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-Associated Molecular Pattern
PR	Pathogenesis-related
PCR	Polymerase Chain Reaction
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PTI	PAMP-triggered immunity
RT-qPCR	quantitative Reverse Transcription Polymerase Chain Reaction.
RB	Right border
RdR6	RDR6 RNA-dependent RNA polymerase 6
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SA	Salicylic acid
SAR	Systemic acquired resistance
SD	Standard deviation
SOD	Superoxide dismutase
sRNA	Small RNA
siRNA	Small interfering RNA
UTR	Untranslated region
WT	Wild type

Summary

Plants have an innate immunity system that allows them to defend themselves against infection by pathogens. The defense response involves an important reprogramming of gene expression in the plant and transcriptional activation of genes coding for: i) Proteins and compounds with antimicrobial activity; ii) Regulatory proteins of defense response (i.e. transcription factors and other transcription regulators, protein kinases, etc.); and iii) proteins/enzymes implicates in hormone signaling associated with the defense response. The current view of the immune response of plants is that, in addition to the mechanisms of transcriptional regulation of gene expression in the plant defense response, also involves mechanisms of post-transcriptional regulation that are regulated by small RNAs. microRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level by degradation or translational repression of their target genes. The regulatory function of miRNAs in processes associated with plant growth and development is widely described. However, available information about the involvement of miRNAs in plant defense against pathogens is more limited.

This thesis comprises the study of miRNAs in innate immunity in plants. The work has been developed in rice (Chapter I and Chapter II) and in Arabidopsis (Chapter III), model systems used in studies of functional genomics in monocotyledonous and dicotyledonous species, respectively. Chapter I describes the functional identification and characterization of new rice miRNAs in their interaction with the fungus *Magnaporthe oryzae*. This fungus is responsible for blast disease, one of the most devastating diseases for rice cultivation worldwide. From the information generated by high-throughput sequencing of small rice RNA libraries, candidate sequences to represent novel rice miRNAs were selected. In this work 5 of these candidates have been studied (miR-64, miR-75, miR-96, miR-98 and miR-203). Obtaining transgenic rice lines has demonstrated that the overexpression of *MIR-64* and *MIR-75* confers resistance to *M. oryzae*, therefore these miRNAs function as positive regulators in the rice immune response. Moreover, overexpression of *MIR-96*, *MIR-98* or *MIR-203* increase susceptibility to *M. oryzae* in rice plants (negative regulators of immune response). Analysis of rice mutants affected in the miRNA biogenesis (*dcl1*, *dcl3* and *dcl4* mutants) indicate that the mature miRNA production of miR-64, miR-75 or miR-96 depends on DCL3 and/or DCL4, which supports the idea that they are novel rice miRNAs. Furthermore, by gene editing using CRISPR/Cas9, it has been found that a 22

nucleotides deletion in miR-75 precursor results in a susceptibility phenotype under *M. oryzae* infection (Chapter II), in agreement with a resistance phenotype that was observed in overexpressor plants for this miRNA.

In chapter III, the miR858 function in *Arabidopsis thaliana* innate immunity to infection by pathogenic fungi was studied. This miRNA represses the expression of *MYB* transcription factors, which act as activators of the expression of genes involved in flavonoids biosynthesis. Plants are resistant to infection by pathogenic fungi (*Plectosphaerella cucumerina*, *Fusarium oxysporum* f. sp. *Conglutinans* and *Colletotrichum higginsianum*) when the activity of miR858 is blocked by the expression of *target mimicry* (*MIM858* plants), while the overexpression of this miRNA confers greater susceptibility to infection. Additionally, interference with miR858 activity and consequent increase of *MYB* gene expression in *MIM858* plants significantly affects phenylpropanoids metabolism, favoring the synthesis and accumulation of flavonoids, and disfavoring the synthesis of lignin precursors. The antifungal activity that was observed for Kaempferol, naringenin (flavonoids) and p-Coumaric acid, would explain the resistant phenotype by fungi infection which is observed in the *MIM858* plants.

Altogether, the results obtained in this work demonstrate that miRNAs are an important component in the resistance/susceptibility to infection by pathogenic fungi in Arabidopsis and rice plants. Greater knowledge of miRNA function in plant innate immunity and processes that are regulate by these riboregulators, can be useful in the design of new strategies for the control of diseases in plants.

Resumen

Las plantas poseen un sistema de inmunidad innata que les permite defenderse frente a la infección por patógenos. La respuesta de defensa implica una reprogramación importante de la expresión génica en la planta y activación transcripcional de genes que codifican para: i) proteínas y compuestos con actividad antimicrobiana; ii) proteínas reguladoras de la respuesta de defensa (p.e. factores de transcripción y otros reguladores de la transcripción, proteínas quinasa, etc); y iii) proteínas/enzimas implicados en la señalización por hormonas asociadas a la respuesta de defensa. La visión actual de la respuesta inmune de las plantas es que, además de los mecanismos de regulación transcripcional de la expresión génica, en la respuesta de defensa de las plantas también participan mecanismos de regulación post-transcriptional que son regulados por pequeños ARNs.

Los microARNs (miARNs) son un clase de pequeños ARNs no codificantes que regulan la expresión génica a nivel post-transcripcional dirigiendo la degradación o la represión traduccional de sus genes diana. La función reguladora de los miARNs en procesos asociados al crecimiento y desarrollo de las plantas está ampliamente descrita. Sin embargo, la información disponible acerca de la implicación de miARNs en la respuesta de defensa de las plantas frente a patógenos es más limitada.

Esta tesis aborda el estudio de miARNs en la inmunidad innata en plantas. El trabajo se ha desarrollado en arroz (Capítulo I y Capítulo II) y en Arabidopsis (Capítulo III), los sistemas modelo utilizados en estudios de genómica funcional en especies monocotiledóneas y dicotiledóneas, respectivamente. En el capítulo I se describe la identificación y caracterización funcional de nuevos miARNs de arroz en su interacción con el hongo *Magnaporthe oryzae*. Este hongo es responsable de la piriculariosis, una de las enfermedades más devastadoras para el cultivo del arroz a nivel mundial. A partir de la información generada mediante secuenciación masiva de bibliotecas de pequeños ARNs de arroz, se seleccionaron secuencias candidatas a representar nuevos miARNs de arroz, habiéndose estudiado 5 de estos candidatos (miR-64, miR-75, miR-96, miR-98 y miR-203). La obtención de líneas transgénicas de arroz ha permitido demostrar que la sobreexpresión de *MIR-64* y *MIR-75* confiere resistencia a *M. oryzae*, tratándose por lo tanto de miARNs que funcionan como reguladores positivos en la respuesta inmune de arroz. Por otra parte, la sobreexpresión de *MIR-96*, *MIR-98* o *MIR-203* aumenta la

susceptibilidad a la infección por *M. oryzae* en plantas de arroz (reguladores negativos de la respuesta inmune). El análisis de mutantes de arroz afectados en la biogénesis de miARNs (mutantes *dcl1*, *dcl3* y *dcl4*) indican que la producción del miARN maduro miR-64, miR-75 o miR-96 es dependiente de DCL3 y/o DCL4, lo cual apoya la idea de que se trata de nuevos miARNs de arroz. Además, mediante edición génica por CRISPR/Cas9, se ha comprobado que una delección de 22 nucleótidos en el precursor miR-75 resulta en un fenotipo de susceptibilidad a *M. oryzae* (Capítulo II), lo que concuerda con el fenotipo de resistencia que se observa en las plantas que sobreexpresan este miARN.

En el capítulo III se ha estudiado la función de miR858 en la inmunidad innata de *Arabidopsis thaliana* frente a la infección por hongos patógenos. Este miARN reprime la expresión de factores de transcripción de tipo *MYB* que actúan como activadores de la expresión de genes que participan en la biosíntesis de flavonoides. Cuando la actividad del miR858 se encuentra bloqueada por la expresión de un gen de imitación de díana (plantas *MIM858*), las plantas son resistentes a la infección por hongos patógenos (*Plectosphaerella cucumerina*, *Fusarium oxysporum* f. sp. *Conglutinans* and *Colletotrichum higginsianum*), mientras que la sobreexpresión de este miARN confiere mayor susceptibilidad a la infección. Además, la interferencia con la actividad de miR858, y consiguiente aumento de la expresión de genes *MYB*, en las plantas *MIM858* afecta de manera importante el metabolismo de fenilpropanoides, priorizándose la síntesis y acumulación de flavonoides, a expensas de la síntesis de precursores de lignina. La actividad antifúngica que se observa para kaempferol, naringenina (flavonoides) y ácido p-cumárico, explicaría el fenotipo de resistencia a la infección por hongos que se observa en las plantas *MIM858*.

En su conjunto, los resultados obtenidos en este trabajo demuestran que los miARNs son componentes importantes en la resistencia/susceptibilidad a la infección por patógenos fúngicos en plantas de arroz y Arabidopsis. Un mayor conocimiento de función de miARNs en la inmunidad innata de las plantas, y de los procesos que son regulados por estos riboreguladores, puede ser de utilidad en el diseño de nuevas estrategias para el control de enfermedades en plantas.

GENERAL INTRODUCTION

1. El arroz.

El arroz, es un alimento básico para más de la mitad de la población mundial, siendo el tercer cereal más cultivado después del maíz y del trigo (FAOSAT, 2016) (Figura 1A). El 90% de la producción total de arroz se encuentra en Asia. En los últimos diez años se ha aumentado la superficie cultivada de arroz (en un 8%) y su producción se ha incrementado en un 22% lo que corresponde a más de 700 millones de toneladas de arroz (FAOSAT, 2016).

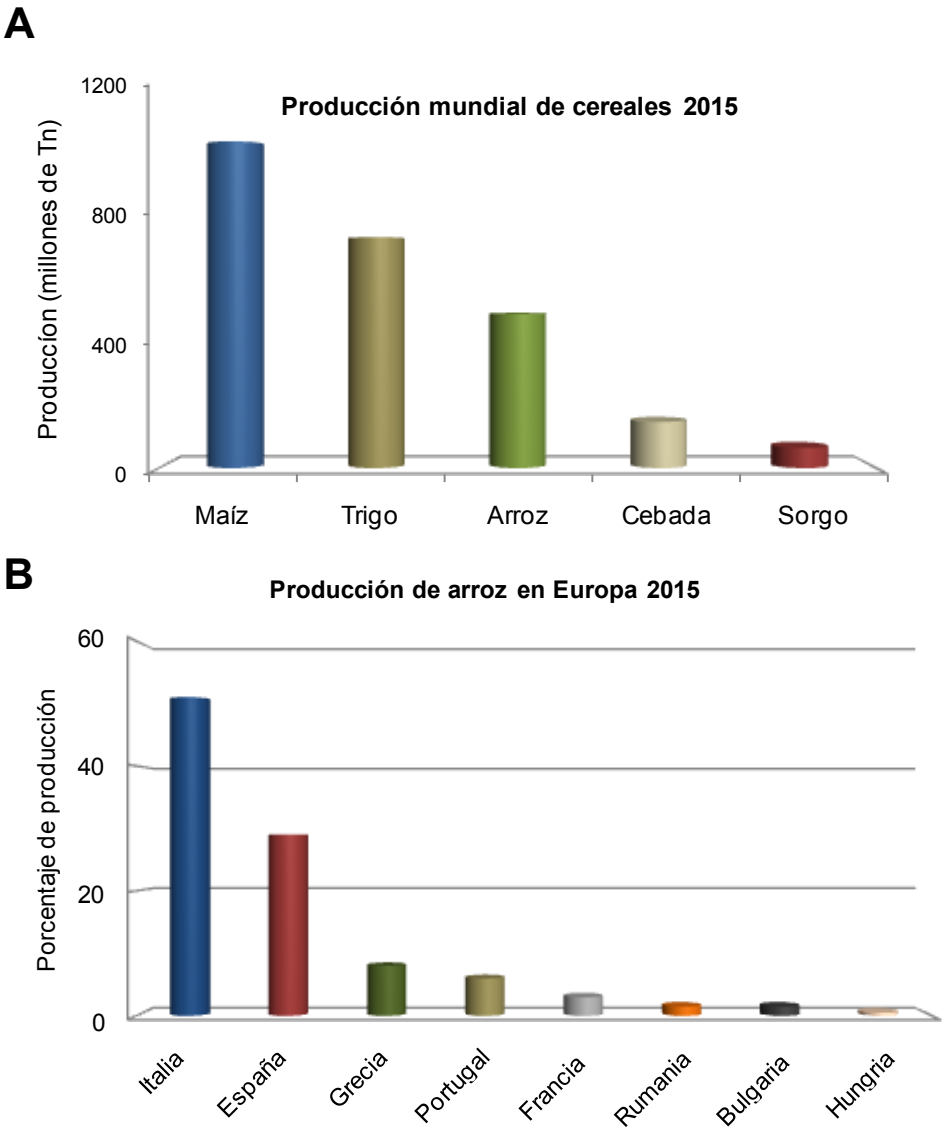


Figura 1. Producción de cereales a nivel mundial **(A)** y de arroz en Europa **(B)** en 2015.

El arroz se cultiva en más de 100 países del mundo. China, India, Indonesia, Bangladesh y Vietnam son los mayores productores de arroz (FAOSTAT, 2016). Fuera

del continente asiático, Brasil es el país con mayor producción. En Europa, se destinan aproximadamente 450.000 hectáreas al cultivo del arroz, siendo Italia el primer productor de arroz europeo (aprox. 50% de la producción) (Figura 1B) (FAOSTAT, 2016). España es el segundo país en producción de arroz en Europa, cultivándose también en Francia, Grecia, Portugal, Bulgaria, Hungría y Rumania. La producción media anual de arroz en Europa es de 3,1 millones toneladas, sin embargo no es suficiente para abastecer a toda su población y se requiere importar arroz para su consumo en Europa (aprox. 1,1 millones de toneladas).

1.1. La planta de arroz.

El arroz (*O. sativa* spp.) es una planta angiosperma monocotiledónea, perteneciente a la familia de las gramíneas (*Poaceae*). Los caracteres morfológicos y fisiológicos del arroz se pueden diferenciar durante el desarrollo de la planta, en sus etapas de crecimiento vegetativo o reproductiva: germinación, producción de tallos secundarios, emergencia de panículas, floración y maduración (Figura 2). La morfología de la planta de arroz es similar a la de otras plantas gramíneas. El tallo se forma por unidades de brotes que están compuestos de nodos e internodos. Las hojas se encuentran unidas al tallo por una vaina basal enrollada en un cilindro que envuelve las hojas de nueva formación. El arroz posee raíces fibrosas y cubiertas de pelos radiculares, con una raíz principal y varias raíces secundarias (Figura 2). Las especies de arroz silvestres muestran diversidad en sus rasgos morfológicos, como altura, tallo, hojas, floración, panículas, semillas, entre otros, con respecto a las especies cultivadas (Zhang and Wing, 2013).

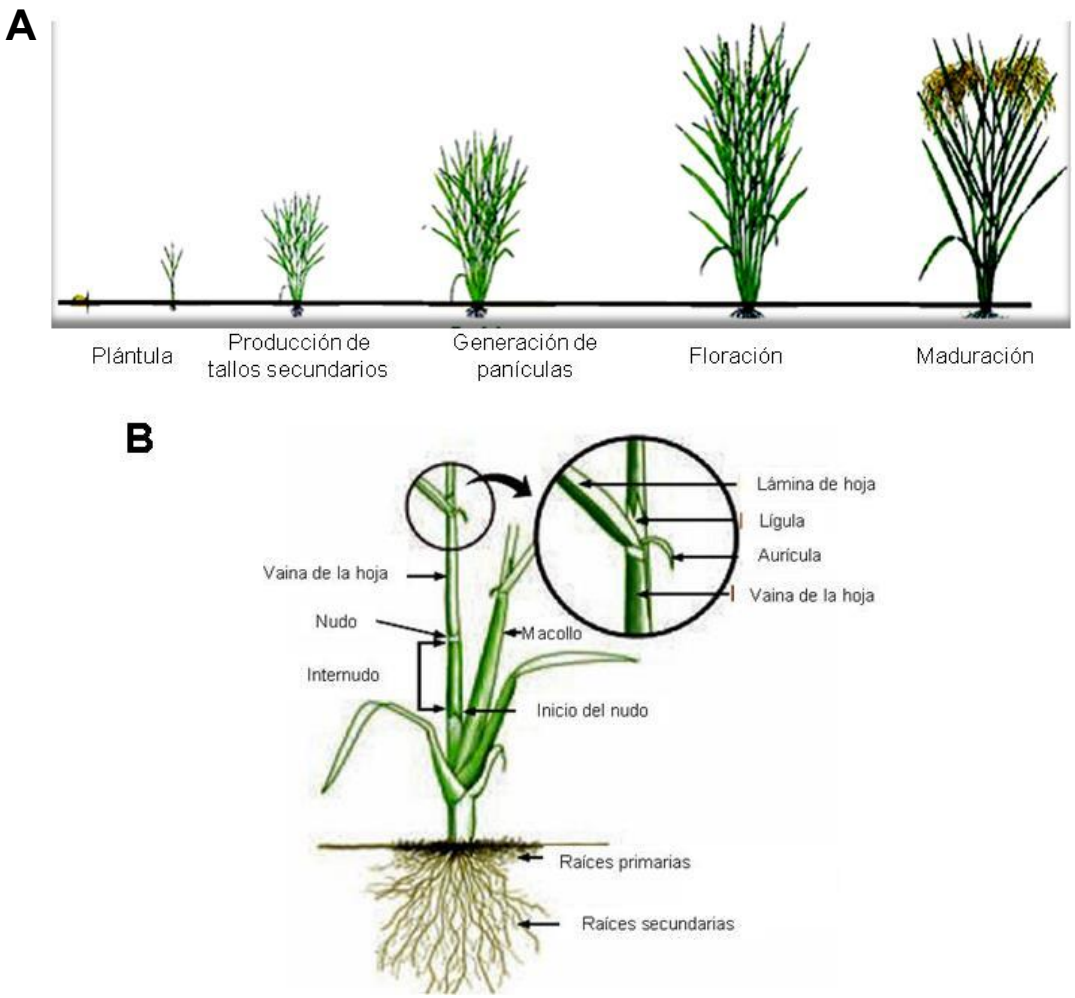


Figura 2. Morfología de la planta de arroz. **(A)** Estadios de desarrollo **(B)** Partes de la planta de arroz. Adaptado de Maclean, et al., 2013.

En la actualidad el arroz se cultiva en una amplia gama de ambientes y es productivo en suelos donde otros cultivos no lo son. Se cultiva en ecosistemas muy diferentes, en climas tropicales y templados, desde el nivel del mar hasta altitudes de 2.000m. Según las condiciones de irrigación, el arroz se cultiva en parcelas inundadas (*paddy*) con inundación periódica y en suelo (no inundado). La producción es mayor en climas cálidos y húmedos, en parcelas con inundación permanente (Figura 3).

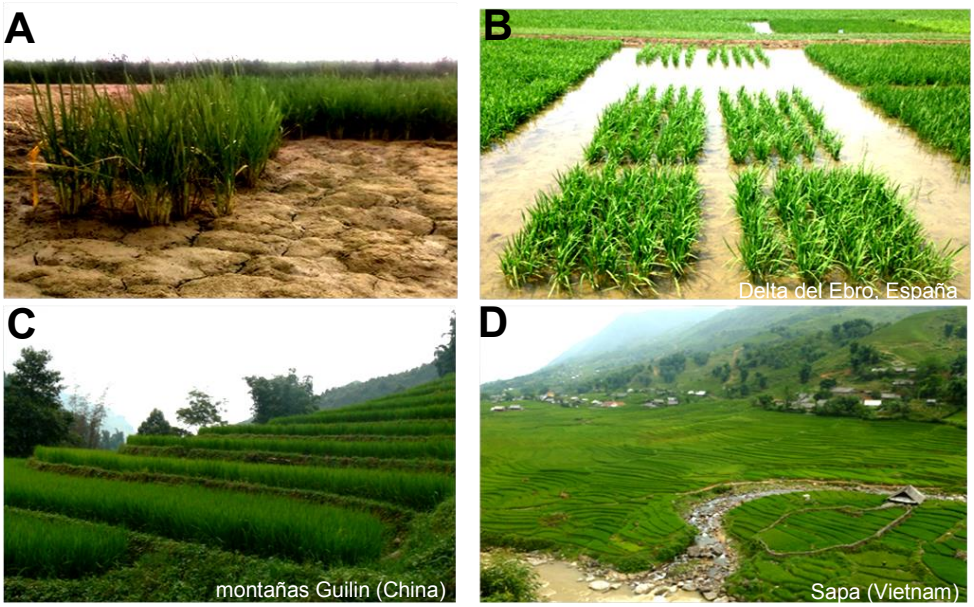


Figura 3. Ecosistemas en el cultivo de arroz. **(A)** Cultivo en secano **(B)** Parcelas inundadas **(C)** Cultivo en terrazas **(D)** Cultivo en parcelas con irrigación.

El género *Oryza* incluye 2 especies cultivadas, *O. sativa* (arroz asiático) y *O. glaberrima* (arroz africano), y 22 especies silvestres (Sanchez et al., 2014; http://www.gramene.org/species/oryza/rice_intro.html). El arroz asiático es el más cultivado a nivel mundial. Dentro de las especies silvestres del género *Oryza* se encuentran *O. officinalis*, *O. punctata*, *O. rhizomatis*, *O. rufipogon*, *O. nivara*, *O. meridionalis*, *O. alta*, *O. granulata*, *O. Ridleyi*, *O. brachyantha*, *O. barthii* y *O. latifolia*, entre otras. La domesticación de *O. sativa* ocurrió hace aproximadamente 10.000 años, por lo que el cultivo del arroz tiene un largo historial de selección natural y domesticación. Se considera que los ancestros de *O. sativa* son *O. nivara* (anual) y *O. rufipogon* (perenne) (Sweeney and McCouch, 2007; Dogara and Jumare, 2014). El arroz africano (*O. glaberrima*) se originó de manera independiente a partir de un ancestro silvestre, *O. barthiii* en la región del Delta del río Níger en África Occidental, con posterioridad a la domesticación del arroz asiático (Wang et al., 2014).

Además, *O. sativa* contiene subespecies que pertenecen a los grupos *japonica* e *indica*. Las variedades *japonica* fueron domesticadas en la región del río Pearl en el sur de China, mientras que las variedades *indica* se desarrollaron posteriormente a partir de cruces entre el grupo *japonica* y especies de arroz silvestre locales durante el proceso de expansión en Asia del sur (Huang et al., 2012). A su vez, *O. sativa japónica* se diferenció en las subespecies "*temperate japónica*" (comúnmente denominadas

japónica) y "*tropical japónica"* (*javanica*) (Garris et al., 2005). Las subespecies del tipo *japonica* producen granos redondos, y son las más ampliamente cultivadas en Europa.

O. sativa contiene 12 pares de cromosomas, es diploide (2n=24), su genoma tiene un tamaño de aproximadamente 430 Mb, con alrededor de 40.000 genes codificantes para proteínas. Se trata por lo tanto de un genoma relativamente pequeño, de tamaño 3 veces superior al de la planta modelo de Arabidopsis (135 Mb) (Goff, 2002; Yu et al., 2002).Dentro del género *Oryza*, se encuentran representados diferentes tipos de genoma tanto diploides (AA, BB, CC, EE, FF y GG) como tetraploides (BBCC, CCDD, KKLL y HHJJ) (Figura 4).

Las dos especies cultivadas, *O. sativa* (asiática) y *O. glaberrima* (africana) poseen genoma del tipo AA. En 2002, se publicó la secuencia del genoma de dos subespecies de *O. sativa*, del grupo *japónica* (cv. *Nipponbare*) y del grupo *indica* (cv. 9311) (Goff, 2002; Yu et al., 2002). La re-secuenciación del genoma de *O. sativa* (cv. *Nipponbare*) mediante el sistema Illumina permitió generar una secuencia de referencia para el genoma de *O. sativa japonica* (*Os-Nipponbare*-Reference-IRGSP-1.0 genome; Kawahara et al., 2013).Se dispone asimismo de la secuencia del genoma del arroz africano (*O. glaberrima*) (Wang et al., 2014). Recientemente el proyecto "Los 3,000 genomas de arroz" (*3,000 Rice Genomes Project*, 3K RGP), llevado en colaboración por investigadores de la Academia China de Ciencias Agrícolas (CAAS), el Instituto Internacional de Investigación del Arroz (IRRI) y el Instituto de Genómica de Pekín (BGI), ha permitido obtener la secuencia del genoma de 3.024 variedades de arroz de 89 países (Li et al., 2014).

La disponibilidad de mapas físicos de alta densidad genética, y el avance en los programas de secuenciación de genomas, muestran que el genoma del arroz tiene una gran sintenia con el genoma de otros cereales (Bennetzen and Ma, 2003). Existen varias plataformas para poder realizar estudios de genómica comparativa en especies del genero *Oryza*, entre ellas se encuentran "*Oryza Map Alignment*, OMAP" (http://www.omap.org/) y "*Oryza Genome Evolution*, OGEP" (http://www.nsf.gov/awardsearch/showAward?AWD_ID=1026200). Además, se han desarrollado protocolos altamente eficientes para la transformación genética del arroz (Hiei et al., 1994) y se han generado colecciones de cDNA (p.e., *Knowledge-based Oryza Molecular Biological*-KOME, http://cdna01.dna.affrc.go.jp/cDNA/ en Japón). Existen asimismo colecciones de mutantes de arroz en diferentes fondos genéticos,

como son: *Oryza-Tag line* (http://oryzatagline.cirad.fr/), *Taiwan Rice Insertional Mutant* (TRIM, http://trim.sinica.edu.tw/), *Rice Mutant Database* (RMD, http://rmd.ncpgr.cn/), *POSTECH-Rice Insertion Database* (RISD, http://cbi.khu.ac.kr/) y *Rice Tos17 Insertion Mutant Database* (https://tos.nias.affrc.go.jp/), entre otras. Se cuenta también con colecciones de germoplasma de arroz, siendo las más importantes la colección generada en el Instituto Internacional de Investigación del Arroz (IRRI, *International Rice Research Institute*, Los Banos, Filipinas; http://irri.org/) y en el Instituto Nacional de Ciencias Agrobiológicas (NIAS, *National Institute of Agrobiological Sciences*, Tsukuba, Japón; http://www.nias.affrc.go.jp/index_e.html). Así pues, el arroz, además de ser uno de los cultivos más importantes a nivel mundial, es la especie modelo para estudios de genómica funcional en plantas monocotiledóneas (cereales), por la comunidad científica internacional.

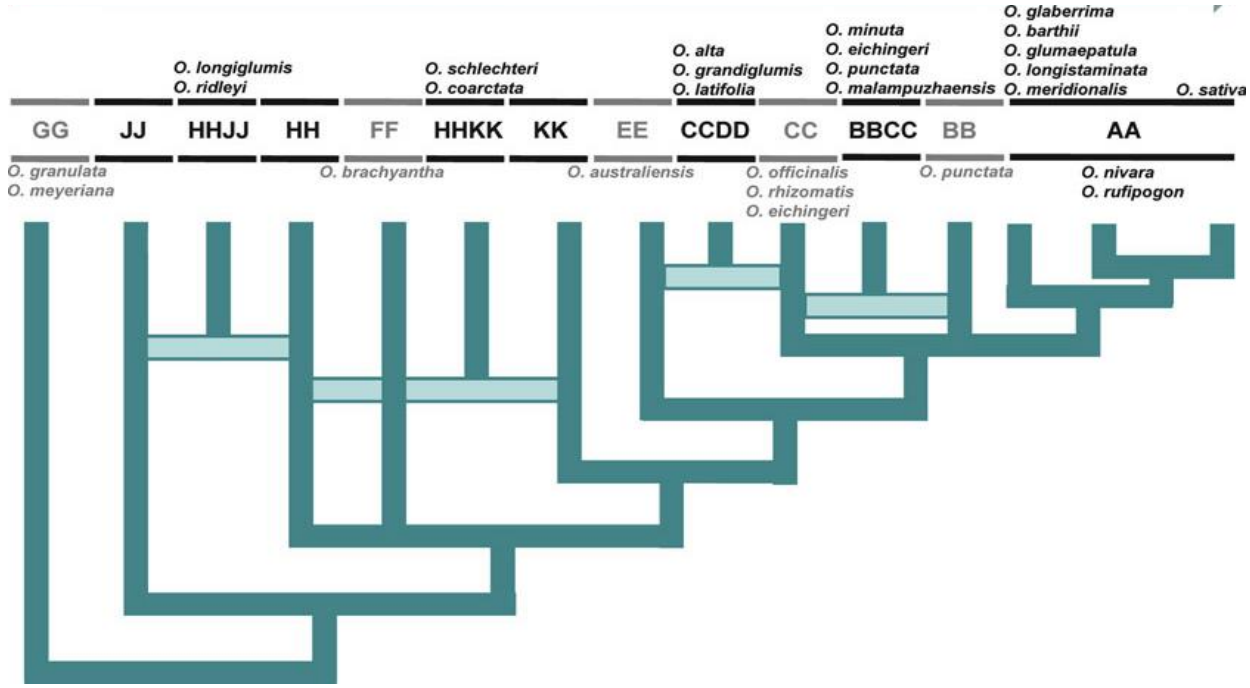


Figura 4. Relación filogenética de diferentes especies del genero Oryza. Tomado de Zhang and Wing, 2013.

Por otra parte, el continuo crecimiento que se observa en la población mundial hace que sea necesario desarrollar estrategias que permitan aumentar la producción de arroz para poder satisfacer la creciente demanda de este alimento. La urbanización y contaminación hace que la disponibilidad de suelos cultivables se encuentre cada vez más limitada. Asimismo, es necesario mantener la producción con un menor suministro de agua y limitando el uso de pesticidas y fertilizantes. El reto, es por lo tanto, poder

aumentar el nivel de producción de arroz en los próximos años, en condiciones cada vez más restrictivas.

1.2. Enfermedades del cultivo de arroz.

El cultivo del arroz se encuentra afectado por diferentes microorganismos (virus, hongos, bacterias), así como también por insectos y plantas parásitas, que son responsables de importantes pérdidas económicas a nivel mundial. Las enfermedades causadas por hongos y bacterias tienen más importancia en cuanto a perdidas en la producción de arroz. La FAO estima que las pérdidas en las cosechas por enfermedades causadas por estos patógenos son del 20% al 40% de la producción mundial.

El patógeno más devastador para el arroz es el hongo *Magnaporthe oryzae*, responsable de la enfermedad de la piriculariosis (descrito en el apartado 1.3). El segundo patógeno fúngico de más importancia a nivel de daños en los cultivos de arroz es *Rhizoctonia solani*, que causa la enfermedad del añublo de la vaina del arroz o "*sheath blight*" (Yellareddygari et al., 2014) (Figura 5A). La enfermedad conocida como "*bakanae*" (*foolish seedling*) es causada por especies del género *Fussarium spp*. (forma telemórfica *Gibberella fujikuroi*), principalmente *F. fujikuroi*, *F. proliferatum* y *F. verticillioides* (Wulff et al., 2010) (Figura 5B). Las plántulas de arroz que han sido infectadas por este hongo presentan un crecimiento superior al normal debido a la producción de giberilina por el hongo (hormona implicada en el crecimiento de la planta) (De Datta, 1981). Esta enfermedad hace que la mayoría de las plantas afectadas mueran antes de generar panículas.

Determinadas bacterias también son patogénicas para el arroz, como es el caso de *Xanthomonas oryzae* (Mew et al., 1993) que causa la bacteriosis vascular del arroz (*rice bacterial blight*) (Figura 5C). La infección por la bacteria *Dickeya dadantii* (anteriormente, *Erwinia chrysantemi*) da lugar a la podredumbre blanda o marchitamiento (*soft rot*) (Goto, 1979). Finalmente, la bacteria *Burkholderia glumae* es responsable de la enfermedad del añublo bacterial de la panícula (Ham et al., 2011).

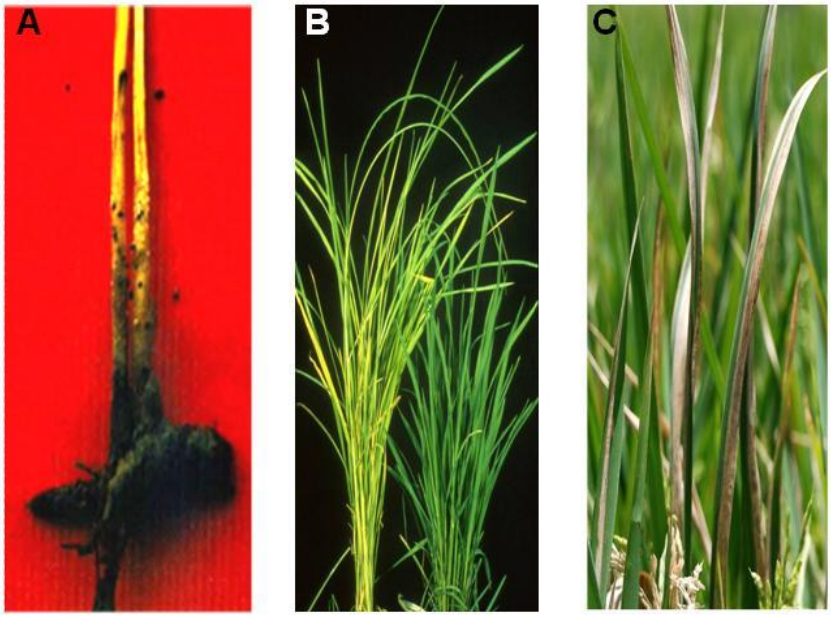


Figura 5. Enfermedades del arroz. **(A)** Enfermedad del añublo de la vaina del arroz (*sheath blight*), causada por *Rhizoctonia solani.* Tomado de Rondoy M., 2009. **(B)** Enfermedad del "*bakanae*" (*foolish seedling*), producida por especies del género *Fussarium spp.* (IRRI, http://www.knowledgebank.irri.org/). **(C)** Bacteriosis vascular del arroz (*rice bacterial blight*), causada por *Xanthomona oryzae*. (IRRI, http://www.knowledgebank.irri.org/).

Mientras que las enfermedades causadas por hongos o bacterias tienen una distribución mundial, las enfermedades causadas por virus en arroz se encuentran delimitadas a zonas geográficas concretas. En el caso del virus de la hoja blanca (*Rice Hoja Blanca Virus,* RHBH) (Morales and Niessen, 1983), este virus se encuentra en América del Sur, mientras que el virus del moteado amarillo del arroz (*Rice Yellow Mottle Virus,* RYMV) (Allarangaye et al., 2006), es un patógeno importante en Africa. En cuanto a los virus que afectan los cultivos de arroz en Asia se encuentran: el virus de la raya del arroz (*Rice Stripe Virus,* RSV), el virus del arroz enano (*Rice Dwarf Virus,* RDV) y los virus tungro del arroz (*Rice Tungro Viruses,* RTBV y RTSV) (Uehara-Ichiki et al., 2013).

El cultivo del arroz también se encuentra seriamente afectado por plagas de insectos, entre los cuales cabe mencionar el barrenador del arroz (*Chilo suppressalis*, lepidóptero). Recientemente, un molusco gasterópodo el caracol manzana (*apple snail*, *Pomacea insularum*) ha sido incorporado a la lista de agentes dañinos para el cultivo de arroz. Los caracoles del genero *Pomacea* son nativos de los humedales de Sudamérica y son considerados especies exóticas invasoras importantes. En Europa, el caracol manzana fue detectado por primera vez en 2009 en el Delta del Ebro, donde fue introducido de manera accidental, y en la actualidad representa un grave problema en los campos de arroz de esta región.

1.3. *Magnaporthe oryzae* y la piriculariosis del arroz.

La enfermedad más devastadora para el cultivo del arroz a nivel mundial es la piriculariosis, causada por el hongo *Magnaphorte oryzae*. Este patógeno puede infectar a la planta en diferentes estadios del desarrollo, hojas, tallos, nudos, y panículas, y en condiciones de laboratorio también infecta a la raíz de la planta (Figura 6) (Bonman et al., 1989; Dufresne and Osbourn, 2001; Sesma and Osbourn, 2004; Wilson and Talbot, 2009). *M. oryzae*, también puede afectar a otros cultivos como por ejemplo la cebada o el trigo. La enfermedad de la piriculariosis, fue descrita por primera vez en el año 1637 en China y fue denominada "enfermedad de la fiebre del arroz". En la actualidad es responsable de pérdidas millonarias en la mayoría de los países productores de arroz (Dean et al., 2012).

Figura 6. La piriculariosis "*Blast disease*" del arroz. Síntomas de Infección por el hongo *M. oryzae* en diferentes partes de la planta de arroz. Fuentes (IRRI, http://www.knowledgebank.irri.org/ y http://www.ehinga.org/).

M. oryzae es un hongo ascomiceto hemibiótrofo. El ciclo infectivo de *M. oryzae* ha sido estudiado principalmente en hoja (Figura7) (Wilson and Talbot, 2009). La infección por *M. oryzae* se inicia cuando el conidio (tricelular) se adhiere a la superficie de la hoja gracias a la producción de un adhesivo mucilaginoso que es secretado por el compartimento apical de la espora durante la hidratación. En el extremo apical se genera un tubo germinativo que crece sobre la superficie cuticular y genera el apresorio, estructura especializada necesaria para la penetración en el tejido huésped. En el espacio entre la pared celular y la membrana plasmática del apresorio se deposita melanina que ayuda a mantener la integridad del apresorio.

La acumulación de glicerol en el apresorio permite generar la fuerza necesaria para la penetración de la pared celular de la célula vegetal a través de una hifa especializada (*penetration peg*) que surge de la base del apresorio (Howard and Valent, 1996). Una vez en el interior de la célula epidérmica, las hifas crecen y se ramifican formando las hifas primarias (finas y cortas), características de la etapa biotrófica de crecimiento del hongo en la planta huésped.

Posteriormente, las hifas adquieren una morfología diferente, siendo más gruesas y bulbosas (estado necrotrófico del hongo). El hongo invade las células contiguas a través de los plasmodesmos (Kankanala et al., 2007; Khang et al., 2010; Campos-Soriano et al., 2013). Los síntomas de la piriculariosis son evidentes 3–4 días después de la infección. Se trata de lesiones necróticas romboidales con bordes de color marrón y centros de color grisáceo. Con el tiempo, las lesiones se agrandan y se agrupan hasta ocupar casi por completo la superficie de la hoja. En condiciones ambientales idóneas (p.e. alta humedad), el hongo esporula y las esporas son dispersadas por la lluvia o el viento a plantas vecinas.

En 2012, se describió que la piriculariosis del arroz es la enfermedad más importante causada por hongos en plantas (Dean et al., 2012). Se dispone de la secuencia del genoma de *M. oryzae* (Dean et al., 2005) así como también de colecciones de mutantes *(Agrobacterium Tumerfaciens-Mediated Transformation Database for M. oryzae*, ATMT http://atmt.snu.ac.kr/; *Magnaporthe grisea*/*Oryza Sativa*, MGOS, *Interaction Database: Community Annotation,* http://www.mgosdb.org/information.html). La interacción arroz/*M. oryzae* resulta por tanto un modelo de estudio de interés, no solo desde el punto de vista científico sino también desde una perspectiva socio-económica.

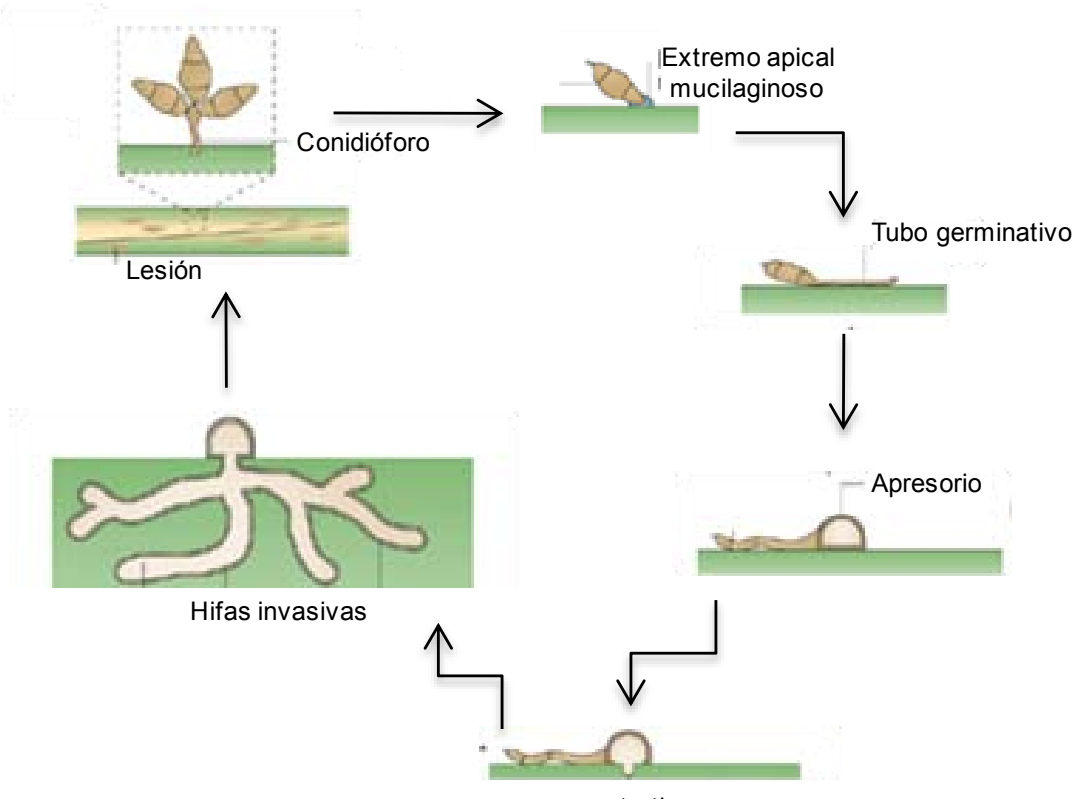


Figura 7. Ciclo infectivo de *M. oryzae* en el arroz. Figura adaptada de Wilson and Talbot, 2009.

2. *Arabidopsis thaliana.*

2.1. La planta de Arabidopsis.

Arabidopsis thaliana (2n=10) es una planta dicotiledónea perteneciente a la familia *Brassicaceae.* Tiene un ciclo de vida corto y se encuentra distribuida por todo el mundo. En cuanto a sus características fisiológicas y morfológicas, es una planta de tallo erecto con ramificaciones y hojas de dos tipos: las basales, formando una roseta, y las hojas caulinares (en el tallo, más pequeñas y carentes de pecíolo). Las inflorescencias se forman en el extremo de las ramas y tallo, y se van separando unas de otras a medida que el tallo crece (Figura 8).

Las flores de Arabidopsis son hermafroditas con los órganos florales claramente diferenciados y normalmente con cuatro pétalos blancos. El fruto es una silicua alargada, de forma cilíndrica y ligeramente arqueada con dos cavidades en las que se alojan las semillas. Las semillas son ovoideas, se disponen en una hilera y están separadas entre ellas. Una silicua puede contener en su interior aproximadamente 30 semillas.

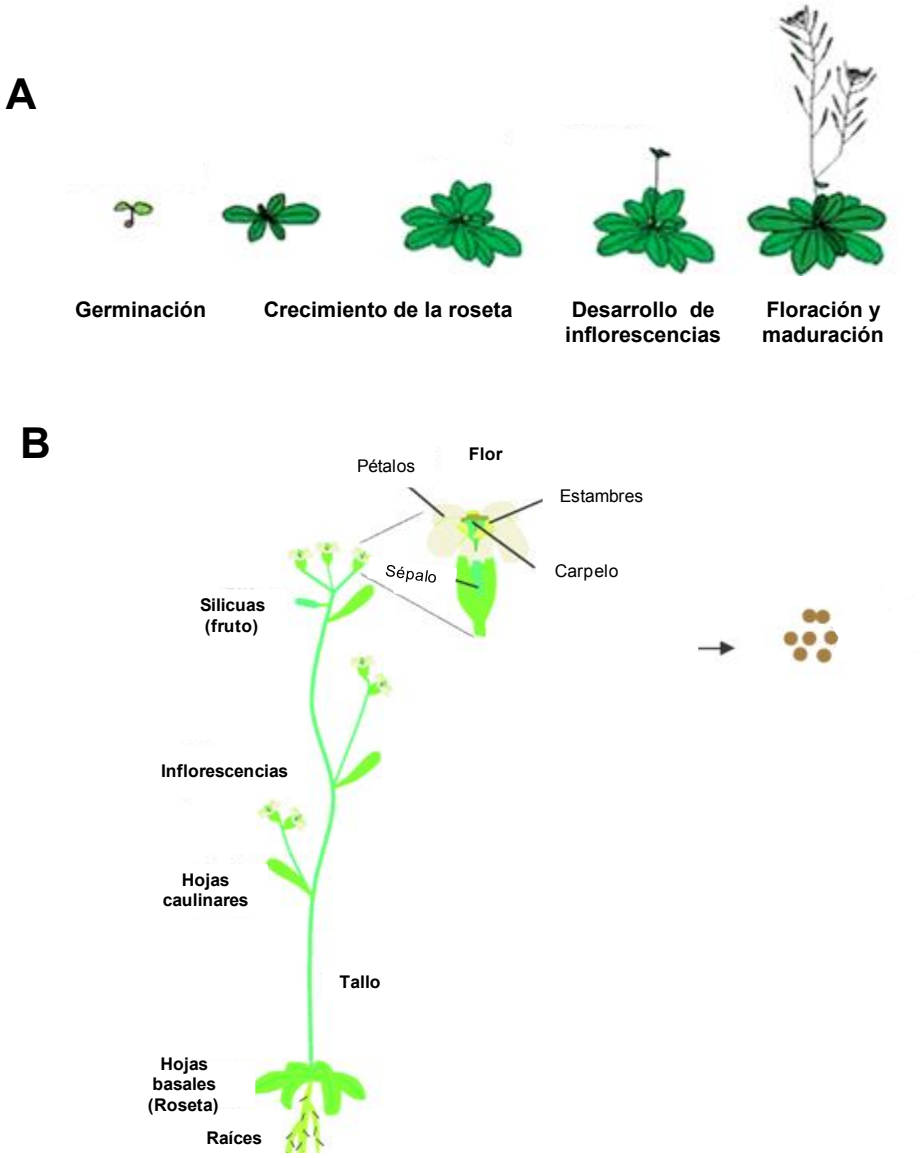


Figura 8. Morfología de la planta de Arabidopsis. **(A)** Estadios de desarrollo de Arabidopsis **(B)** Partes de la planta de Arabidopsis. Adaptado de Diévart and Clark, 2004 y Shindo, et al., 2007.

El proyecto "Iniciativa para el Genoma de Arabidopsis" (*Arabidopsis Genome Initiative*, AGI) permitió que *Arabidopsis thaliana* se convirtiera en la primera planta con su genoma secuenciado. *A. thaliana* tiene un genoma pequeño (135 Mb) distribuido en cinco cromosomas. Se cuenta con una colección de aproximadamente 1500 accesiones recolectadas de todo el mundo, que están disponibles en bancos de semillas, el principal es el *Arabidopsis Biological Resource Center* (ABRC) (distribuidas por *The Nottingham Arabidopsis Stock Centre*, NASC). La existencia de un elevado número de mutantes, protocolos de transformación eficientes y bases de datos disponibles (*The Arabidopsis*

Information Resource, TAIR), ha permitido que la planta *A. thaliana* sea la planta modelo para estudios en dicotiledóneas (Mitchell-Olds, 2001; Van Norman and Benfey, 2009).
Como hongos patógenos importantes para *Arabidopsis thaliana*, además de *Plectosphaerella cucumerina* (apartado 2.2) cabe citar *Botrytis cinérea* (necrótrofo) (Williamson et al., 2007), *Colletotrichum* spp y *Fusarium oxysporum* f. sp. *conglutinans* (hemibiótrofos) (Mauch-Mani and Slusarenko, 1994; O'Connell et al., 2004). En cuanto a bacterias patogénicas para *A. thaliana*, la más ampliamente utilizada es *Pseudomonas syringae* (Katagiri et al., 2002).

2.2. *Plectosphaerella cucumerina*.

Plectosphaerella cucumerina, (anteriormente, *Fusarium tabacinum*; forma anamórfica, *Plectosporium tabacinum*) es un hongo ascomiceto necrótrofo responsable de la enfermedad de tizón (*Blight disease*) en diferentes especies vegetales (p.e. melón, la rúcula, canónigos, bambú, entre otros) (Palm et., 1995; Carlucci et al., 2012; Giraldi, et al., 2013). *P. cucumerina* puede sobrevivir saprofíticamente en material vegetal en descomposición del suelo.
La interacción *A. thaliana/P. cucumerina* ha surgido como un modelo de interés para el estudio de la respuesta de defensa de las plantas frente al ataque de hongos necrótrofos (Berrocal-Lobo et al., 2002; Ton and Mauch-Mani, 2004; Ramos et al., 2013).

3. Inmunidad innata en las plantas.

Las plantas son capaces de desarrollar una respuesta de defensa frente a organismos potencialmente patogénicos, o inmunidad innata. En general, las plantas presentan un primer nivel defensivo basado en la existencia de barreras físicas preexistentes (pared celular) que dificultan la penetración del patógeno, y en la acumulación de compuestos que pueden ser tóxicos para el patógeno. Estos mecanismos son constitutivos y pueden no ser suficientes para detener la invasión del patógeno. Así pues, las plantas también desarrollan mecanismos de defensa activos que son inducidos por la presencia del patógeno.

3.1. Inmunidad activada por el reconocimiento PAMPs (PTI) e inmunidad activada por el reconocimiento de efectores (ETI).

Las plantas perciben la presencia de patógenos a través del reconocimiento de patrones moleculares del patógeno (*Pathogen-Associated Molecular Patterns,* PAMPs) por receptores de la planta (*Pattern Recognition Receptors,* PRRs) (Jones and Dangl, 2006; Zipfel, 2008; Boller and He, 2009). Este reconocimiento desencadena una respuesta en la planta que se ha denominado inmunidad activada por PAMPs (*PAMP-triggered immunity,* PTI) o defensa basal. Además, la planta puede reconocer moléculas propias, como son fragmentos de la pared que son liberados tras la acción de enzimas producidas por los patógenos. El reconocimiento de patrones moleculares asociados al daño (*Damaged-associated molecular pattern,* DAMPs), también activa la respuesta de defensa de la planta (Boller and Felix, 2009; Tanaka et al., 2014).

Determinados patógenos a su vez han desarrollado mecanismos para superar este primer nivel de defensa con la producción de compuestos capaces de interferir con la respuesta PTI, conocidos como efectores (Figura 9). Así mismo, las plantas pueden reconocer directamente o indirectamente dichos efectores a través de otro tipo de receptores, las proteínas de resistencia (proteínas *R*). Este reconocimiento es específico de huésped y patógeno y desencadena una respuesta denominada inmunidad activada por efectores (*Effector-Triggered Immunity,* ETI) que es altamente efectiva para contrarrestar el ataque del patógeno (Dangl and Jones, 2001; Chisholm et al., 2006; Pritchard and Birch, 2014) (Figura 9). La respuesta ETI va frecuentemente asociada a la respuesta hipersensible (*Hypersensitive Response*, HR) en la cual se produce la muerte celular controlada (*Programmed Cell Death,* PCD) en el sitio de la infección, que limita la propagación de la infección (Jones and Dangl, 2006).

Las proteínas *R* poseen dominios conservados. El grupo de proteínas *R* más común presenta un dominio de unión a nucleótidos (*Nucleotide-Binding Site,* NBS) en la región central y un dominio de repeticiones ricas en leucinas (*Leucin-Rich Repeat,* LRR) en su extremo C-terminal. En la región N-terminal se encuentra, bien un dominio de tipo *coiled-coil* (CC), o bien dominios con similitud a las proteínas Toll de Drosophila e interleukinas de mamíferos (las denominadas proteínas CC-NBS-LRR y TIR-NBS-LRR, respectivamente) (Hammond-Kosack and Kanyuka, 2007). Otro tipo de proteínas *R* se caracteriza porque sus miembros poseen solamente un dominio LRR extracelular, como

las proteínas de la familia Cf de tomate (*Solanum lycopersicum*) que confieren resistencia al hongo *Cladosporium fulvum* (Stergiopoulos et al., 2010).

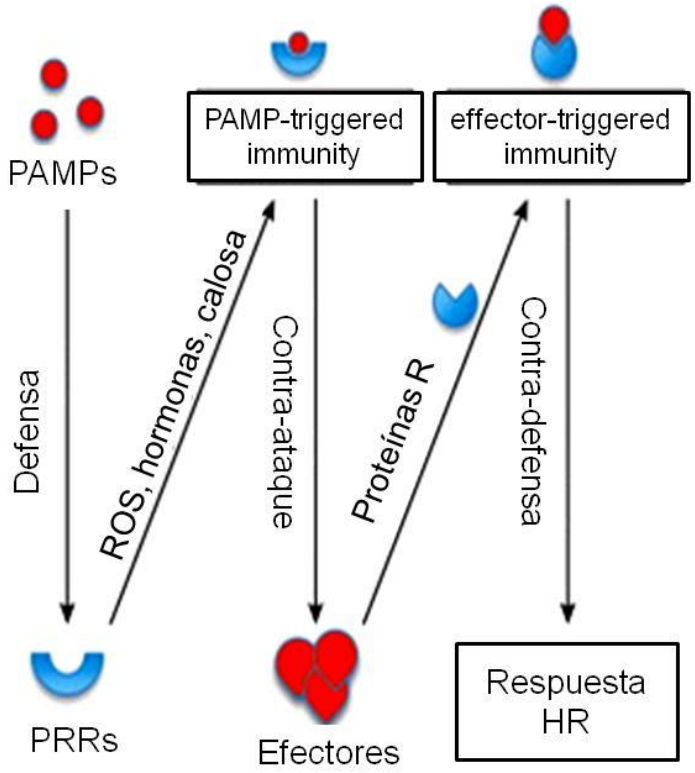


Figura 9. Mecanismos de defensa en las plantas. Modelo adaptado de Jones and Dangl 2006.

El modelo "zigzag" definido por Jones y Dangl (2006) ha sido ampliamente utilizado para explicar los procesos que se observan durante la interacción planta/patógeno, si bien, más recientemente se han descrito limitaciones en este modelo (Pritchard and Birch, 2014). Se trata de un modelo que es aplicable fundamentalmente a la interacción con patógenos biótrofos, en el que no se consideran respuestas "cuantitativas". En este modelo tampoco se considera la respuesta a DAMPs, ni tampoco el hecho de que el resultado de la interacción planta-patógeno (resistencia o susceptibilidad) puede estar influenciado por factores ambientales. Se requiere, por tanto, desarrollar modelos más dinámicos que incluyan factores que pueden ser determinantes para la resistencia o susceptibilidad a la infección por patógenos en plantas (Pritchard and Birch, 2014).

En el arroz, la resistencia a patógenos bacterianos y fúngicos es conferida tanto por genes de resistencia (ETI) como por la resistencia inducida por PAMPs (PTI) (Liu et al., 2014). Se han descrito numerosos genes de resistencia a *M. oryzae* en arroz que se utilizan con éxito en programas de mejora genética (Wang et al., 1999; Bryan et al.,

2000; Qu et al., 2006; Ashikawa et al., 2008; Ballini et al., 2008; Liu et al., 2014). Sin embargo la resistencia a *M. oryzae* mediada por genes de resistencia no es duradera (aprox. 2 años) debido al rápido cambio en la patogenicidad del hongo y diversidad de aislados de este patógeno en campo. Ello ha llevado al desarrollo de programas de mejora basados en la incorporación de múltiples genes de resistencia (*pyramiding*) en variedades cultivadas de arroz (Fukuoka et al., 2015).

3.2. Mecanismos y rutas de señalización en la respuesta de defensa.

El reconocimiento del patógeno por la planta desencadena una serie de respuestas rápidas, como son la despolarización de la membrana plasmática y alteraciones en el flujo de iones a través de ella (entrada de H^+ y Ca^{2+}; salida de K^+ y Cl^-). La entrada de Ca^{2+} (segundo mensajero en muchos procesos celulares), activa rutas de señalización en las que participan proteínas quinasa, como son las proteínas quinasa dependientes de calcio (*Calcium-dependent protein kinases*, CPKs) (Harper et al., 1993; Romeis et al., 2001). También se han descrito proteínas quinasa activadas por mitógenos (*Mitogen-Activated Protein Kinases*, MAPKs) implicadas en la respuesta de defensa de las plantas (Romeis et al., 2001; Pitzschke et al., 2009; Meng and Zhang, 2013; Bigeard et al., 2015).

Otros fenómenos que se activan rápidamente en respuesta a la infección son la producción de especies reactivas de oxígeno (*Reactive Oxygen Species*, ROS) y de óxido nítrico (*Nitric Oxide*, NO) (Mellersh et al., 2002; Torres et al., 2006; Liu et al., 2010; Bellin et al., 2013; Baudouin and Hancock, 2014; Baxter et al., 2014). Si bien las plantas producen ROS continuamente como subproducto de procesos metabólicos, en situación de infección se produce una “explosión oxidativa” (*oxidative burst*) como parte de la respuesta de defensa (Wojtaszek, 1997; Bolwell et al., 2002; Morel et al., 2004). Las moléculas ROS tienen una doble función, pudiendo actuar como agentes antimicrobianos y como moléculas señalizadoras para la activación de respuestas de defensa. Entre ellas, cabe mencionar el radical superóxido (O_2^-), peróxido de hidrógeno (H_2O_2) y radical hidroxilo (OH^-). La acumulación de H_2O_2 además favorece la polimerización de precursores de lignina en la pared celular (lignificación) y dificulta la penetración del patógeno (Figura 10).

Sin embargo, cuando se encuentran en exceso las moléculas ROS pueden resultar tóxicas para la propia planta, dada su capacidad para modificar de manera irreversible

lípidos (p.e. lípidos de membrana), proteínas y ácidos nucleicos, con el consiguiente daño celular. Por ello, las plantas poseen mecanismos para mantener los niveles adecuados de ROS en la célula y evitar así el daño oxidativo en los que participan compuestos con capacidad antioxidante (glutatión, ascorbato, flavonoides, alcaloides, carotenoides, tocoferoles) y actividades enzimáticas (glutatión-S-transferasas, superóxido dismutasas, catalasas, peroxidasas, etc). Las moléculas ROS, junto con el óxido nítrico, potencian la respuesta hipersensible (Levine et al., 1994; Delledonne et al., 2001; Lin et al., 2012).

Además de los programas de muerte celular, el óxido nítrico participa en otros procesos asociados a la defensa, interaccionando con rutas de transducción en las que participan proteínas quinasas y hormonas señalizadoras en defensa (ácido salicílico, ácido jasmónico), y en la movilización de mensajeros secundarios (p.e. Ca^{2+}) (Garcia-Mata and Lamattina, 2002; Pagnussat et al., 2004; Courtois et al., 2008).

La deposición de callosa y la acumulación de proteínas ricas en hidroxiprolina en la pared celular, son respuestas frecuentes de la planta por la presencia del patógeno que contribuyen a dificultar la penetración del patógeno en el tejido al que infecta (Luna, 2011; Ellinger et al., 2014). En situación de infección, la planta también activa la producción de metabolitos secundarios, como es el caso de las fitoalexinas (ver apartado 3.2.1). Algunos de estos compuestos presentan actividad antimicrobiana (Galeotti et al., 2008; De Conti Lourenço et al., 2013; Mierziak et al., 2014).

Se han descrito factores de transcripción pertenecientes a diferentes familias que regulan la expresión de genes de defensa (Li et al., 2016). Entre ellos se encuentran factores de transcripción *WRKY*, que reconocen cajas de tipo W (*W-box*) (Lai et al., 2008; Pandey and Somssich, 2009), los factores de transcripción *AP2/ERF* (asociados principalmente a la ruta de señalización del etileno) (Oñate-Sánchez et al., 2007; Pré et al., 2008), factores de transcripción *bZIP* (*Basic Leucine Zipper Domain*, y factores de transcripción *MYB* (Raffaele and Rivas, 2013; Shan et al., 2016).

Como resultado de la activación de la compleja red de interacciones entre distintas rutas de señalización, se observa la inducción de la expresión de genes que codifican para proteínas relacionadas con patogénesis (*Pathogenesis-Related*, *PRs*). Las proteínas *PR* se han clasificado en diferentes familias atendiendo a su homología de secuencia y función de las mismas (Van Loon, 1985; Van Loon and Van Strien, 1999). Determinadas

proteínas *PR* presentan actividad antimicrobiana (p.e. quitinasas, β-1,3-glucanasas, defensinas, y tioninas, entre otras) (Figura 10).

Las hormonas tienen un papel fundamental en la regulación de la respuesta de defensa de las plantas, concretamente el ácido salicílico (*Salicylic Acid*, SA), el ácido jasmónico (*Jasmonic Acid*, JA y jasmonatos) y el etileno (*Ethylene*, ET) (Glazebrook, 2005; Bari and Jones, 2009; Robert-Seilaniantz et al., 2011; Denance et al., 2013; De Vleesschauwer et al., 2014). El ácido abscísico (*Abscisic Acid*, ABA) también regula la inmunidad innata en muchos tipos de interacción planta/patógeno, tambien en *A. thaliana/P. cucumerina* (Sánchez-Vallet et al., 2012). Históricamente, el ácido salicílico se ha asociado a la defensa de las plantas frente al ataque por patógenos biótrofos, mientras que el ácido jasmónico y el etileno se asocian con la defensa frente a patógenos necrótrofos (Glazebrook, 2005). Además, el ácido jasmónico participa en la regulación de la respuesta frente a insectos herbívoros (Farmer et al., 2003; Onkokesung et al., 2010; Machado et al., 2016). Las rutas del ácido salicílico y del ácido jasmónico/etileno son mutuamente antagonistas. Sin embargo, tambien se han descrito interacciones sinérgicas entre estas vías, lo cual sugiere que la red de señalización utilizada por la planta es dependiente tanto del estilo de vida del patógeno en la planta huésped, como de la planta huésped en sí misma.

La señalización mediada por SA participa en la respuesta hipersensible que se observa durante la infección por biótrofos (Eneydi et al., 1992; Vlot et al., 2009). Uno de los componentes clave en la vía de señalización del SA es la proteína *NPR1* (*non-expressor of PR genes 1*), capaz de interaccionar con factores transcripcionales del tipo TGA que reconocen elementos reguladores en *cis* en el promotor de genes de defensa, como el gen *PR1* (Cao et al., 1998; Spoel et al., 2003, 2007). Las auxinas, los brasinoesteroides y el ácido giberélico (gibberelic acid) también son hormonas señalizadoras en defensa (Albrecht et al., 2012; De Vleesschauwer et al., 2012) (Figura 10).

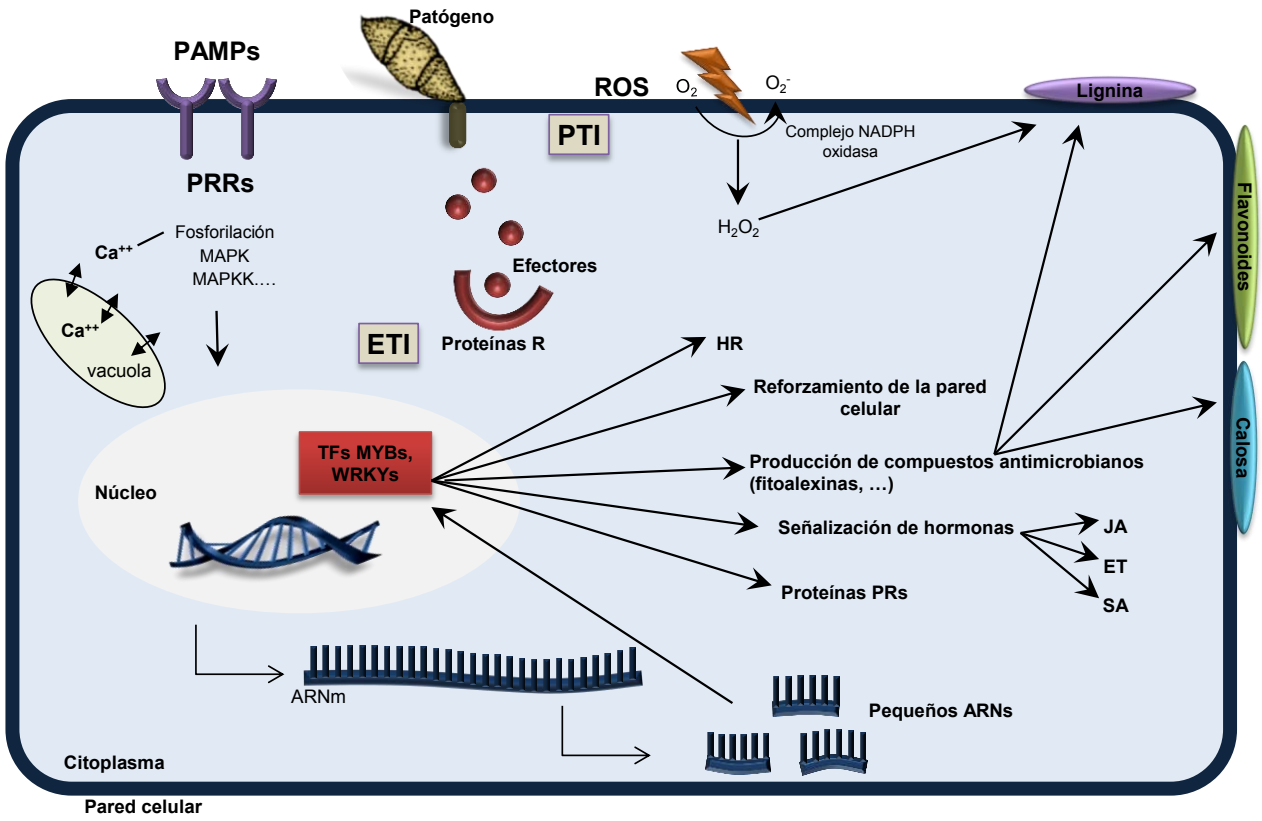


Figura 10. Inmunidad innata en las plantas. Mecanismos implicados en la respuesta de defensa de las plantas frente a la infección por patógenos. PAMPS (*Pathogen-Associated Molecular Patterns*); PRRs (*Pattern Recognition Receptors*); PTI (*PAMP-triggered immunity*); ETI (*Effector-Triggered Immunity*); *PRs* (*Pathogenesis-Related*); HR (*Hypersensitive Response*); MAPK (*Mitogen-Activated Protein Kinase*); MAPKK (*Mitogen-Activated Protein Kinase Kinase*); JA (*Jasmonic Acid*); ET (*Ethylene*); SA (*Salicilic acid*); TFs (*Transcription Factor*); ARNm (ARN mensajero); ROS (*Reactive Oxygen Species*); NADPH oxidasa (*Nicotinamide Adenine Dinucleotide Phosphate-oxidase*).

Las plantas también desarrollan mecanismos de resistencia a nivel sistémico entre los que se encuentran la resistencia sistémica adquirida (*Systemic Acquired Resistance*, SAR) (Ryals et al., 1996; Durrant and Dong, 2004) y la resistencia sistémica inducida (*Induced Systemic Resistance*, ISR) (Van Loon et al., 1998; Pieterse et al., 2014). La SAR es inducida tras una infección inicial por un patógeno, se manifiesta en sitios distantes al foco de infección, y es efectiva para contrarrestar la infección no solo por el patógeno que la desencadenó inicialmente sino también por otros patógenos (Cao et al., 1998). La respuesta SAR puede mantenerse durante largos periodos de tiempo (hasta semanas) y va acompañada de un aumento de la hormona ácido salicílico (SA) en tejidos sistémicos. La mayor acumulación de SA induce la expresión de genes de defensa como son los genes relacionados con patogénesis (*Pathogenesis-Related*) o genes *PR*.

La ISR se induce por microorganismos del suelo que infectan la raíz de las plantas (Pieterse et al., 1996; Van Loon et al., 1998). Al igual que la SAR, la ISR es una resistencia sistémica, de amplio espectro que depende de cascadas de señalización inducidas por hormonas, como son el etileno (ET) y ácido jasmónico (JA). La ISR va acompañada de la activación de genes relacionados con patogénesis (*Pathogenesis-Related*) *PRs*. El tratamiento con determinados agentes químicos (p.e. ácido amino butírico, BABA y el benzotiodiazol, BTH) también puede activar mecanismos de defensa típicamente asociados a la resistencia inducida (Jakab et al., 2001; Kohler et al., 2002; Ton and Mauch-Mani, 2004).

Hace más de diez años que se describió el fenomenon "*priming*" en la defensa de las plantas (Conrath et al., 2006, 2015; Martinez-Medina et al., 2016). El fenómeno descrito como *priming* induce un estado fisiológico, en el cual la planta está condicionada para la activación de la defensa de una manera más rápida, más intensa o más duradera. El *priming*, o potenciador de la defensa se ha desarrollado como rasgo adaptativo para el ajuste de la defensa de la planta en ambientes impredecibles. Es una medida defensiva adaptativa y de bajo costo, ya que no depende de la expresión constitutiva de mecanismos de defensa con el consiguiente coste energético que ello representa para la planta. El *priming* se observa en diferentes situaciones: resistencia sistémica adquirida, tratamiento con determinados compuestos (BABA, liposacáridos bacterianos), interacción con microorganismos beneficiosos (p.e. hongos micorrícicos, rizobacterias), así como también por alteraciones en el metabolismo. En estudios recientes se ha observado que el *priming* de defensa puede pasar de una generación a la otra, lo que indica la implicación del *priming* como un componente epigenético de la defensa transgeneracional (Luna et al., 2012).

Los estudios moleculares acerca del fenómeno del *priming* de defensa se han asociado a cambios en la cromatina, y la acumulación de ARNm de genes con un papel de señalización en defensa (p.e. reguladores transcripcionales, proteínas quinasas, receptores de reconocimiento de patrones) (Conrath, 2011; Singha et al., 2013; Duan et al., 2014). En situación de infección, la planta responde de manera más rápida e intensa, permitiendo así una respuesta más eficaz para contrarrestar la infección por el patógeno (Delaney, 1997; Ebrahim et al., 2011; Oliveira et al., 2016). Sin embargo la información de que se dispone actualmente sobre los mecanismos moleculares subyacentes al *priming* y su importancia en la resistencia de las plantas es bastante limitada.

3.2.1. Metabolitos secundarios en la respuesta de defensa de las plantas.

Las plantas producen una gran cantidad de metabolitos secundarios muy diversos en su estructura química, que potencialmente pueden participar en la defensa frente a patógenos. Probablemente el ejemplo más estudiado es la producción de fitoalexinas en Arabidopsis. La fitoalexina indólica más abundante en Arabidopsis es la camalexina, sintetizada a partir del triptófano (Schuhegger et al., 2006). La contribución de camalexina, y otros derivados del triptófano que en términos generales se conocen como indol-glicosinolatos, en la respuesta de defensa de las plantas ha demostrada en diferentes patosistemas (Thomma et al., 1999; Bednarek et al., 2009; Sánchez-Vallet et al., 2010; Schlaeppi et al., 2010; Stotz et al., 2011; Bednarek, 2012).

Los flavonoides representan otro grupo importante de metabolitos secundarios en plantas, y se sintetizan a partir del aminoácido fenilalanina, a través de la ruta de los fenilpropanoides "*phenylpropanoid metabolic pathway*". Arabidopsis produce tres grupos principales de flavonoides: flavonoles, antocianinas y proantocianinas. La primera enzima de la ruta de los fenilpropanoides es la fenilalanina amoniaco liasa (*phenylalanine ammonia lyase*, PAL) que cataliza la producción de ácido cinámico quien posteriormente es convertido en ácido p-cumárico y en 4-coumaroil-CoA por la actividad de los enzimas cinamato-4-hidroxilasa (*cinnamic acid 4-hydroxylase*, C4H) y 4-cumarato: Coenzima A ligasa (*4-coumarate-CoA ligase,* 4CL). El fenilpropanoide 4-coumaroil-CoA, es el precursor para la síntesis de flavonoles y antocianinas, así como también para la producción de monolignoles (monómeros de la lignina) (Falcone et al., 2012; Mierziak et al., 2014; Mouradov and Spangenberg, 2014). Para más detalles sobre la ruta de biosíntesis de fenilpropanoides, ver Figure 6 Chapter III. La biosíntesis de flavonoides es un proceso altamente regulado en el que se observan interacciones de diferentes familias de factores de transcripción que participan en las diferentes ramas de esta ruta de manera modular. Entre ellos destacan miembros de la familia *R2R3* de factores de transcripción *MYB*, factores de transcripción *bHLH* (*basic helix-loop-helix*) y *WD40* (Buer et al., 2010; Falcone-Ferreyra et al., 2012; Mouradov and Spangenberg, 2014).

Aunque el mecanismo por el cual los flavonoides participan en la defensa de las plantas frente a al ataque por patógenos no se ha caracterizado en detalle, se ha propuesto que se basa en la capacidad de estos metabolitos para actuar como agentes antioxidantes y/o quelantes de metales capaces de inducir estrés oxidativo en la planta (Van Etten et

al., 1994; Reguant et al., 2000; Dixon et al., 2002; D. Treutter, 2005; Naoumkina et al., 2010; Hendra et al., 2011).

Las fitoalexinas que también presentan una gran diversidad de estructura química, son producidas por las plantas como parte de su respuesta defensiva frente a la infección por patógenos y para algunas fitoalexinas se ha descrito una actividad antimicrobiana (Jeandet et al., 2013, 2014).

4. Pequeños ARNs en plantas.

Durante mucho tiempo se consideró que las respuestas PTI y ETI frente a hongos y bacterias se basaban en la regulación transcripcional de genes codificantes para proteínas (proteínas de defensa) y que estos mecanismos, eran independientes de los mecanismos de silenciamiento de ARN (mecanismos tradicionalmente asociados a la defensa frente a virus). En la actualidad, se sabe que en la respuesta de defensa de las plantas frente a hongos y bacterias también participan mecanismos para la regulación post-transcripcional de la expresión génica que están mediados por la actividad de pequeños ARNs (Navarro et al., 2006; Katiyar-Agarwal and Jin, 2010; Pumplin and Voinnet, 2013; Seo et al., 2013; Weiberg et al., 2014; Fei et al., 2016; Huang et al., 2016; Kuan et al., 2016).

En plantas existen dos clases principales de pequeños ARNs endógenos no codificantes, los microARNs (miARNs) y los pequeños ARNs de interferencia (siRNAs, *small interfering RNAs*). Los miARNs (21-24nt) son ARNs de cadena unica con una función reguladora de la expresión génica a nivel post-transcripcional, bien a través de la degradación de ARN mensajeros (ARNm) diana, o bien por inhibición de su traducción (Llave et al., 2002; Brodersen et al., 2008).

En los últimos años se han empleado técnicas de secuenciación masiva para la identificación de la población de miARNs en muchas especies vegetales (Campo et al., 2013; Lukasik et al., 2013; Shuai et al., 2013; Baldrich et al., 2015). Las secuencias de miARNs se encuentran depositadas en la base de datos "*The microRNA database*, miRBase" (Kozomara and Griffiths-Jones, 2014). El arroz es la especie vegetal con más miARNs anotados en miRBase (713), más que Arabidopsis (427). Sin embargo, son muy pocos los miARNs de plantas para los cuales se dispone de información sobre su función y de los procesos fisiológicos en los que participan (Figura 11).

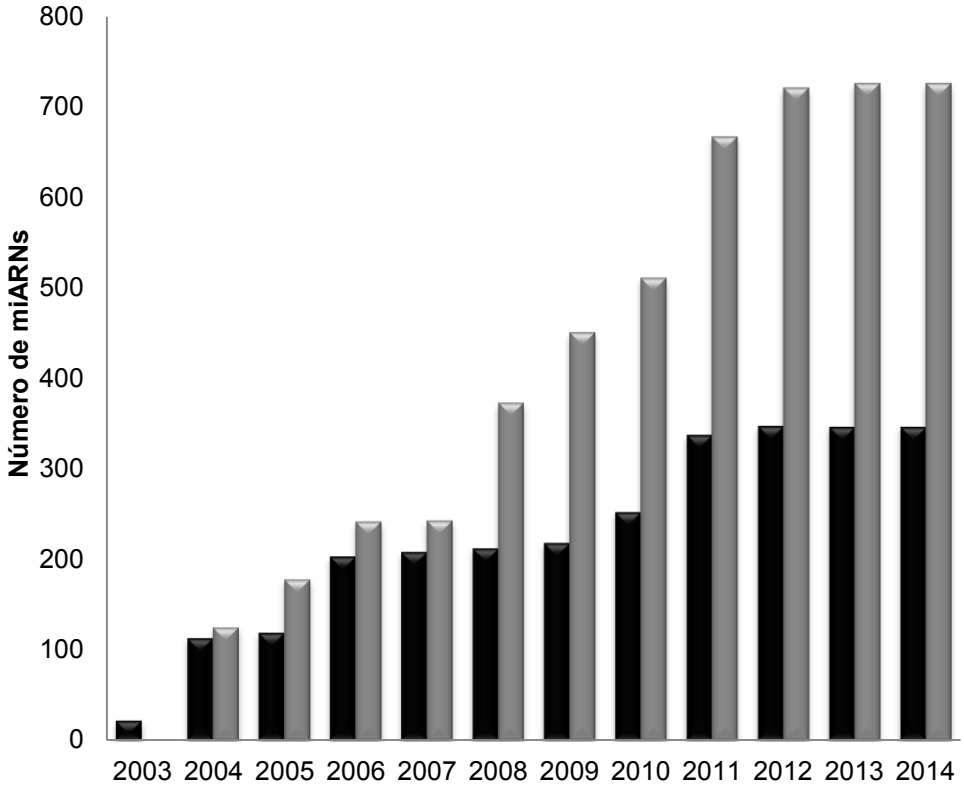


Figura 11. Evolución de la anotación de miARNs en miRBase (*The microRNA database*). Barras negras, número de miARNs maduros anotados para *Arabidopsis thaliana* (427); barras grises, número de miARNs maduros anotados para *O. sativa* (713). Fuente: (http://www.mirbase.org/, versión 2014).

La clase de siRNAs incluye diferentes tipos de pequeños ARNs, como son: *heterochromatic siRNAs* (hc-siRNAs), *natural antisense siRNAs* (nat-siRNAs), *trans-acting siRNAs* (ta-siRNAs), *phased secondary siRNAs* (pha-siRNAs) y *long small interfering RNAs* (lsiRNAs) (Arikit et al., 2013; Axtell, 2013; Fei et al., 2013; Borges and Martienssen, 2015). Las diferencias más importantes entre miARNs y siRNAs residen en el tipo de molécula de la cual proceden (p.e. ARN de cadena sencilla o doble cadena), y en su mecanismo de biogénesis y función.

4.1. Biogénesis y mecanismo de acción de los miARNs en plantas.

La biogénesis de un miARN comienza con la transcripción de un gen nuclear (gen *MIR*) por la ARN *polimerasa II* (Pol II). En la región promotora de genes *MIR* se pueden encontrar las cajas de inicio de la transcripción (tipo TATA) y elementos de regulación característicos de unión a diversos tipos de factores de transcripción (cajas W, MYC, MYB, entre otros), tal y como se encuentra en genes codificantes para proteínas (Zhao and Li, 2013). La mayoría de los genes *MIR* de plantas son intergénicos, si bien también

se han descrito miARNs intragénicos, localizados en intrones de genes codificantes (*mirtrons*).

La transcripción de los genes *MIR* produce un miARN primario (pri-miRNA, *primary-miRNA*) que se encuentra estabilizado por la adición de una metilguanosina (extremo 5'), y una cola poliadenilada (polyA, extremo 3'). Los tránscritos pri-miRNA adoptan una estructura en forma de horquilla que es procesada en dos etapas secuenciales por una ribonucleasa de tipo DICER (*DICER-LIKE*, DCL), generalmente por DCL1, que genera un precursor intermediario (pre-miRNA) y posteriormente un dúplex miRNA-5p/miRNA-3p (también conocido como miRNA/miRNA* dúplex) (Kurihara and Watanabe, 2004) (Figura 12). En la mayoría de los casos, una de las cadenas del miARN dúplex es funcional, mientras que la otra es degradada. Sin embargo, también se han descrito miARNs en los cuales las dos cadenas del dúplex son funcionales (p.e. miR393) (Navarro et al., 2006; Zhan et al., 2011).

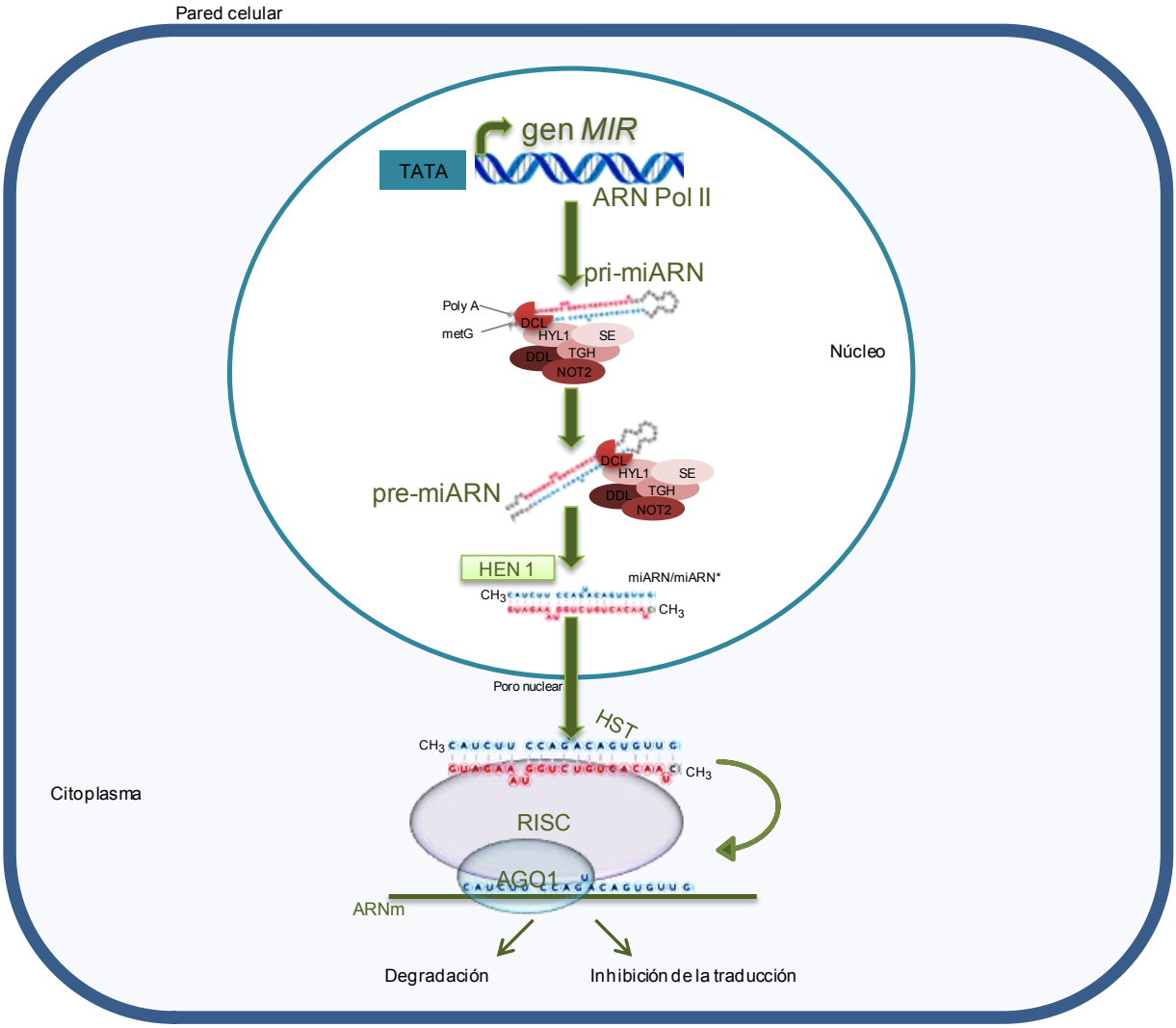


Figura 12. Biogénesis de miARNs. Representación gráfica de la biogénesis y el procesamiento de miRNAs en plantas. ARN POL II (ARN polimerasa II); pri-miARN (miARN primario); pre-miARN (precursor de miARN); DCL(*DICER-LIKE*); HYL1 (*HYPONASTIC LEAVES 1*); NOT2 (*NEGATIVE ON TATALESS 2*); DDL (*DAWDLE*); TGH (*TOUGH*); SE (*SERRATE*); metG (metilguanosina); polyA (cola poliadenilada); HEN 1 (*HUA ENHANCER 1*); HST (*HASTY*); RISC (*RNA-Induced Silencing Complex*); AGO1 (*ARGONAUTE 1*). Adaptado de Voinnet, 2009 y Rogers and Chen, 2013.

Otras proteínas implicadas en el proceso de la biogénesis de los miARNs son: HYL1 (*HYPONASTIC LEAVES 1*), NOT2 (*NEGATIVE ON TATALESS 2*), DDL (*DAWDLE*), TGH (*TOUGH*) y SE (*SERRATE*) (Kurihara et al., 2006; Lobbes et al., 2006; Yang et al., 2006; Dong et al., 2008; Wang et al., 2013).

El dúplex miRNA-5p/miRNA-3p es metilado por la enzima HEN1 (*HUA ENHANCER 1*) y transportado desde el núcleo hasta el citoplasma por proteínas HST (*HASTY*). Una vez en el citoplasma, la cadena del miARN funcional se incorpora en el complejo inductor de silenciamiento RISC (*RNA-Induced Silencing Complex*) en el cual se encuentra la proteína AGO1 (*ARGONAUTA1*) responsable del silenciamiento. El complejo RISC guía al miARN hacia el tránscrito diana (reconocimiento por complementariedad de secuencia). En plantas, la mayoría de los miARNs dirigen la degradación de tránscritos diana en la posición complementaria a los nucleótidos 10-11 del extremo 5' del miARN. Sin embargo, son cada vez más lo ejemplos de inhibición de la traducción por miARNs en plantas (Voinnet, 2009; Li et al., 2013).

En plantas, los genes *MIR* se encuentran distribuidos en todo el genoma (Nozawa et al., 2012). Los miARNs identificados inicialmente en plantas se encuentran muy conservados y también se encuentran conservados su correspondientes genes diana (Jones-Rhoades et al., 2006). Por otra parte, los miARNs de reciente aparición (miARNs jóvenes), suelen tener genes diana no conservados que participan en diferentes procesos biológicos (Zhang et al., 2006; Jones-Rhoades, 2012; Nozawa et al., 2012). Se ha propuesto que la acumulación secuencial de mutaciones en las secuencias de los precursores de miARNs, determina que dichos precursores sean procesados por una u otra proteína DCL (Figura 13). Los miARNs canónicos (21 nucleotidos) son procesados por DCL1, pero también se han descrito miARNs cuyos precursores son procesados por DCL3 o DCL4 (Rajagopalan et al., 2006; Vazquez et al., 2008; Arikit et al., 2013, Zhengrui et al., 2014). La actividad de DCL3 o DCL4 se ha visto en miARNs más largos (long-miRNAs) y en miARNs "jóvenes" (23-25 nucleótidos) desde el punto de vista evolutivo (Figura 13).

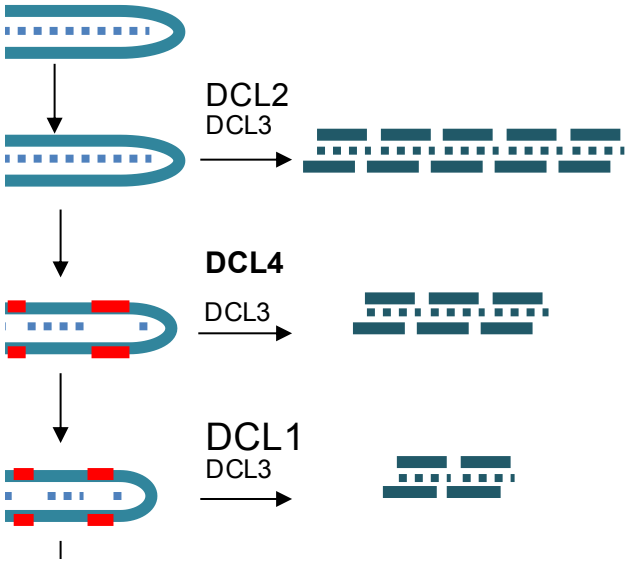


Figura 13. Origen y evolución de los miARNs en plantas. DCL (*DICER-LIKE*); nt (nucleótidos).

A diferencia de los miARNs que se generan a partir de precursores de ARNs de cadena sencilla, los siRNAs endógenos provienen de un ARN de doble cadena que se origina por la actividad de una ARN polimerasa dependientes de ARN (RDR, *RNA-dependent RNA polymerase*) (Chapman and Carrington, 2007; Zhang et al., 2012) (Figura 14). La producción de cada tipo de siRNA a partir del correspondiente precursor requiere la participación de miembros concretos de la familia de proteínas RDR (RDR2, RDR6), y proteínas DCL (DCL2, DCL3 o DCL4). Por ejemplo, los hc-siRNAs requieren RDR2 y DCL3 mientras que la producción de ta-siRNAs requiere RDR6 y DCL4 (Xie et al., 2003; Peragine et al., 2004; Xie et al., 2005; Kasschau et al., 2007). RDR6, DCL1 y DCL2 están implicados en la producción de nat-siRNAs (Borsani et al., 2005; Katiyar-Agarwal et al., 2006; Zhang et al., 2012). Así pues, una diferencia importante entre miARNs y siRNAs es la dependencia de RDR para la producción de siRNAs, pero no para la producción de miARNs.

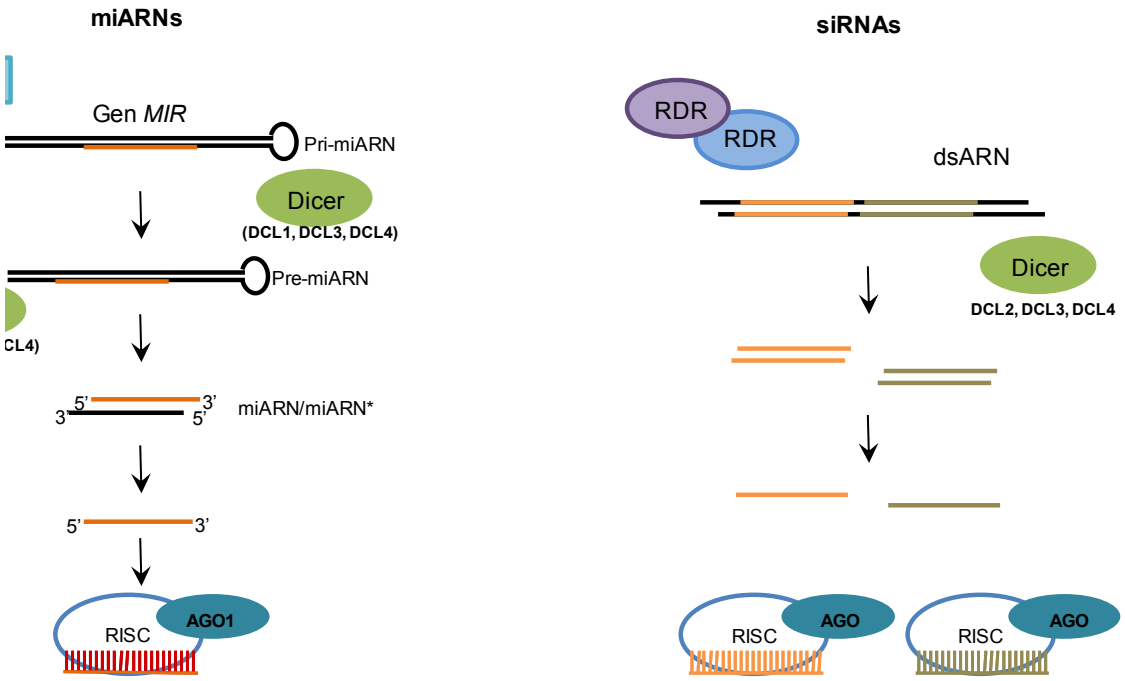


Figura 14. Componentes de la biogénesis de pequeños ARNs en plantas. POL II (ARN Polimerasa II); pri-miARN (miARN primario); pre-miARN (precusor de miARN); DCL (*DICER-LIKE*); RISC (*RNA Silencing Complex*); AGO (*ARGONAUTE*); RDR (*RNA dependent RNA polymerase*); siRNA (*small-interference RNA*); dsARN (*doublé-strand ARN*).

Los siRNAs se incorporan al complejo RISC para realizar su función (Vazquez and Hohn, 2013). Se observa una especificidad en la función de miembros de la familia AGO y de los diferentes tipos de pequeños ARNs. Mientras que AGO1 reconoce preferencialmente miARNs, AGO4 reconoce a los hc-siRNAs (Wang and Axtell, 2016). Los hc-siRNAs (24 nucleótidos) son los siRNAs más abundantes en plantas, y participan en el silenciamiento génico a nivel transcripcional a través de la metilación del DNA dirigida por ARN (RdDM, *RNA-directed DNA methylation*).

4.2. Función de los miARNs.

Los miARNs están involucrados en el control de la expresión génica en procesos asociados al crecimiento y desarrollo de la planta (hojas, flores, raíces), así como también en señalización hormonal, y adaptación a estrés abiótico (sequía, frio, salinidad, estrés oxidativo, o carencia de nutrientes) (Palatnik et al., 2003; Mallory et al., 2004; Miura et al., 2010; Rubio-Somoza and Weigel, 2011; Zhang et al., 2013). Asimismo, se han descrito miARNs que responden a la infección por patógenos (Staiger et al., 2013; Campo et al., 2013; Li et al., 2014; Baldrich and San Segundo, 2016; Soto et al., 2017) En arroz, ciertos miARNs controlan procesos de importancia agronómica como son la

producción de tallos secuntarios (*tillers*), la floración temprana, el desarrollo de las panículas y la producción de granos (Miura et al., 2010; Wang et al., 2012; Zhang et al., 2013).

Los miARNs conservados suelen regular la expresión de genes diana que también se encuentran conservados y presentan niveles de expresión importantes. Muchos de estos miARNs codifican para factores de transcripción implicados en diversos procesos del desarrollo de la planta (Legrand et al., 2010; Ambawat et al., 2013; Lakhotia et al., 2014; Smita et al., 2015). Por otra parte, los miARNs de reciente aparición (miARNs jóvenes) suelen tener genes diana no conservados que codifican para proteínas muy diversas que pueden participar en diferentes procesos biológicos. Estos miARNs jovenes además pueden presentar una expresión espacio-temporal restringida (Campo et al., 2013; Thatcher et al., 2015; Wang et al., 2016).

4.3. miARNs en la inmunidad innata de las plantas.

En los últimos años se ha producido un rápido avance en el conocimiento sobre la biogénesis de miARNs, y mecanismos del reconocimiento miARN-ARNm díana. Sin embargo, la información disponible sobre la función de miARNs en procesos fisiológicos de las plantas, concretamente en inmunidad innata, es muy limitada. Se han identificado miARNs concretos que participan en PTI y/o ETI, en diferentes especies vegetales (Jagadeeswaran et al., 2009a; Padmanabhan et al., 2009; Shivaprasad et al., 2012; Boccara et al., 2014; Gupta et al., 2014; Weiberg et al., 2014; Yang and Huang, 2014; Baldrich and San Segundo, 2016; Niu et al., 2016). A priori, los miARNs inducidos por patógenos podrían modular negativamente la expresión de genes implicados en defensa, mientras que miARNs reprimidos durante la infección podrían ser reguladores positivos de este proceso.

La implicación de los miARNs en la respuesta PTI fue inicialmente demostrada en Arabidopsis. Aquí, el tratamiento de la flagelina (*flagellin22, flg22*), conlleva un aumento en la acumulación de miR393 que reprime la expresión de genes que codifican para receptores de auxina (F-box *auxin receptors*). La represión de la señalización por auxina confiere resistencia a la bacteria *P.syringae* (Navarro et al., 2006). Este estudio estableció un claro vínculo entre la funcionalidad de un miARN, la señalización hormonal y la inmunidad en Arabidopsis. De esta manera, los miARNs que responden a la infección por patógenos, podrían regular la respuesta de defensa de la planta bien de

manera directa, o bien de manera indirecta a través de una regulación hormonal e intercomunicación entre las vías de señalización hormonal relacionadas con la respuesta de defensa. Además, se sabe que las dos cadenas del dúplex mi393/mi393* son funcionales: miR393 regula la vía de señalización por auxinas, y miR393* regula la expresión del gen *MEMB12* que codifica para una proteína SNARE del aparato de Golgi implicada en el transporte vesicular y secreción de la proteína *PR1* (Navarro et al., 2006; Zhang et al., 2011).

En otros estudios se demostró que miR160a es un regulador positivo, mientras que miR398 y miR773 son reguladores negativos de PTI, y que estos miARNs modulan la deposición de calosa en la resistencia frente a *P. syringae* en Arabidopsis (Li et al., 2010). miR398 regula la expresión de transcritos superóxido dismutasa Cu/Zn (*Superoxide Dismutase*, SOD) (*CSD1* y *CSD2*) y para una chaperona de cobre para la superóxido dismutasa. Las proteínas SOD son metaloenzimas que detoxifican ROS, y protegen a las células del estrés oxidativo asociado a la infección por patógenos (Sunkar et al., 2006; Jagadeeswaran et al., 2009b). En otros estudios, se demostró que la sobreexpresión de miR400 o miR844 en Arabidopsis aumenta la susceptibilidad a *P.syringae* (pv. tomato DC3000) y al hongo *Botrytis cinérea* (*B.cinérea*) (Park et al., 2014; Lee et al., 2015). miR400 guía la degradación de transcritos que codifican para proteínas PPR (*Pentatricopeptide Repeat*), mientras que miR844 tiene como diana a transcritos que codifican para CDS3 (*Cytidinephosphate Diacylglycerol Synthase 3*) (Park et al., 2014; Lee et al., 2015).

Se han descrito otros miARNs en solanáceas y leguminosas, así como también en Arabidopsis, que regulan la expresión de genes *R* durante la ETI (Padmanabhan et al., 2009; Jagadeeswaran et al., 2009a; Shivaprasad et al., 2012). En el caso concreto de miR472, este miARN regula respuestas PTI y ETI en Arabidopsis (Boccara et al., 2014), mientras que miR863-3p modula secuencialmente la expresión de genes con una función regulador negativa y positiva de la respuesta de defensa en Arabidopsis (ARLPK, ARLPK2, *atypical receptor-like pseudokinase1/2*) (Niu et al., 2016). Las super familias de miARNs miR482 y miR2118 regulan genes R del tipo *NBS-LRR* en tomate (Shivaprasad, et al., 2012). En tabaco, miR6019 y miR6020 guían la degradación de transcritos del gen *N* (gen de resistencia del tipo TIR-NB-LRR) que confiere resistencia al virus del mosaico del tabaco (*Tobacco Mosaic Virus*, TMV). Para estos miARNs se

sabe que una vez han realizado el corte de su transcrito diana, se producen siRNAs secundarios a partir de sus correspondientes genes *R* diana (Li et al., 2012).

Aunque se ha demostrado que la expresión de una parte importante de la población de miARNs se encuentra regulada por infección en plantas, para la mayoría de ellos no se dispone de información sobre su función en la resistencia a enfermedades. El conocimiento de miARNs implicados en defensa es mucho más limitado que para miARNs relacionados con desarrollo.

4.4. miARNs en la inmunidad innata de arroz.

En los últimos años se han realizado grandes esfuerzos para caracterizar la población de miARNs en diferentes tejidos y/o etapas de desarrollo de la planta de arroz, y en respuesta a estreses bióticos y abióticos. La información obtenida por secuenciación masiva de librerías de pequeños ARNs, ha revelado alteraciones dinámicas en la expresión de un número importante de miARNs de arroz en respuesta a la infección por el hongo *M. oryzae* o al tratamiento con elicitores de este hongo (elicitores crudos) (Campo et al., 2013; Li et al., 2014; Baldrich et al., 2015). La secuenciación masiva de pequeños ARNs también permitió la identificación de miARNs desconocidos hasta entonces, cuya expresión es regulada por tratamiento con elicitores de *M. oryzae* en arroz (Campo et al., 2013; Baldrich et al., 2015).

Hasta la fecha son pocos los miARNs para los que se ha descrito una función en la respuesta de defensa de arroz frente a *M. oryzae*, que son: miR160a, miR398b y miR7695 (Campo et al., 2013, Li et al., 2014). miR160 regula factores de respuesta a auxina (*Auxin Response Factors*, ARFs). Las auxinas tienen un papel crucial en el desarrollo y control de las respuestas inmunes de las plantas tanto en Arabidopsis como en las plantas de arroz (Navarro et al., 2006; Domingo et al., 2009). La sobreexpresión bien de miR160a o de miR398b resulta en una mayor acumulación de H_2O_2 en el sitio de infección y una inducción de la expresión de genes de defensa (p.e. activación de *PR1* y *PR10*) y resistencia a *M. oryzae* (Li et al., 2014).

Resultados anteriores en nuestro grupo de investigación permitieron caracterizar miR7695, que regula negativamente la acumulación de tránscritos del gen *OsNramp6*, (*Natural resistance-associated macrophage protein 6*) (Campo et al., 2013). Las proteínas NRAMP son proteínas transportadoras de metales divalentes a través de membrana (memebrana plasmática, tonoplasto) (Thomine et al., 2003; Cailliatte et al.,

2009; Takahashi et al., 2011). Participan por tanto en la homeostasis de metales en una amplia gama de organismos, incluyendo las plantas. La sobreexpresión de miR7695 en plantas de arroz confiere resistencia a la infección por *M. oryzae* (Campo et al., 2013).

5. Perspectivas y aplicaciones biotecnológicas de los miARNs.

La biotecnología aplicada a la protección de plantas frente a enfermedades representa una estrategia útil para la mejora genética de plantas, complementaria a las técnicas más tradicionales de entrecruzamiento (*breeding*). En la mayoría de especies de interés agronómico se producen grandes pérdidas debido al ataque por patógenos, cuyo control actualmente depende del uso de agentes químicos. No obstante, el uso de productos fitosanitarios tiene consecuencias negativas para el medio ambiente y la salud animal, con la posible aparición de resistencias en la población de microorganismos en campo. Por ello, las directrices de la Unión Europea sobre el uso de plaguicidas (y fertilizantes) limitan cada vez más su utilización. Los métodos de mejora clásica basados en la hibridación sexual, no siempre son posibles, por lo que se hace necesario el desarrollo de nuevas estrategias para el control de enfermedades en plantas.

Tal y como se ha comentado anteriormente, los miARNs han demostrado ser reguladores importantes de caracteres de importancia agronómico y económico. Por ejemplo, se sabe que determinados miARNs controlan la formación de espigas, y por consiguiente, la producción de semillas en la planta de arroz (Miura et al., 2010; Wang et al., 2012). El empleo de tecnologías que permitan silenciar o activar miARNs que regulen genes implicados en la defensa frente a ataques por patógenos, podrían representar herramientas biotecnológicas de gran utilidad para la obtención de resistencia a patógenos en cultivos (Kamthan et al., 2015).

Por otra parte, la utilización de técnicas basadas en la función de miARNs permiten el silenciamiento de genes diana de interés. Este es el caso de los genes miARNs artificiales (amiARNs) o la tecnología de imitación de genes diana (*Target mimicry*). En un gen amiARN la secuencia del miARN maduro de un precursor miARN es sustituida por una secuencia (miARN) diseñada para reconocer un gen diana de interés (Ossowski et al., 2008). El mecanismo de imitación del gen diana es un mecanismo regulador endógeno que las plantas utilizan para regular negativamente la actividad de miARNs específicos (Franco-Zorrilla et al., 2007). Se descubrió por primera vez en el caso del trascrito *IPS1* (*Induced by Phosphate Starvation1*) en el que se encuentra la secuencia

de reconocimiento para el miR399 pero con una mutación en el sitio de corte para miR399. De esta manera, miR399 es secuestrado por *IPS1* impidiendo que este miARN realice su función sobre su transcrito diana (Franco-Zorrilla et al., 2007). La imitación de gen diana podria representar un mecanismo general para la regulación de la actividad de miARNs en plantas. Las tecnologías de silenciamiento de miARNs (imitación de gen diana, miARNs artificiales) podrían, por lo tanto tener aplicaciones importantes para la mejora de la resistencia a enfermedades en plantas.

Por otra parte, la preocupación social sobre del uso de organismos modificados genéticamente (OMG) debe ser tenida en cuenta por la comunidad científica. En esta dirección, es recomendable el uso de nuevas metodologías, que sean “limpias” (p.e. que eviten la integración de ADN foráneo en el genoma de la planta) para la obtención de plantas transgénicas resistentes a enfermedades. Con este fin, las tecnologías desarrolladas para la edición génica y mutagénesis dirigida basadas en el sistema CRISPR/Cas9 (*Clustered Regularly Interspaced Short Palindromic Repeats/Cas9*) pueden resultar de gran utilidad para modificar la expresión de genes *MIR* o de sus genes diana (Miao et al., 2013; Song et al., 2016; Zaidi et al., 2016; Zhao et al, 2016). Esta metodología ha sido utilizada con éxito en arroz (Jiang et al., 2013; Zhang et al., 2014). Más recientemente, esta tecnología está siendo aplicada para deleccionar genes de interés, incluidos genes *MIR* (Zhao et al., 2016).

Por último, los miARNs podrían ser utilizados como biomarcadores para la identificación de variedades resistentes a la infección en poblaciones obtenidas por entrecruzamiento tradicional en programas de mejora genética (Bej and Basak, 2014). Puesto que el arroz es el modelo para estudios de genómica funcional en cereales, el conocimiento adquirido sobre el funcionamiento de miARNs en arroz podría beneficiar a otros cereales de interés.

OBJECTIVES

The general aim of this PhD Thesis was the study of small RNA sequences previously identified in our laboratory by deep sequencing of small RNA populations from rice leaves in the context of rice immunity (*O. sativa/Magnaporthe oryzae* interaction), and to investigate the possible contribution of miR858 in Arabidopsis immunity. The specific objectives of this work were the following:

1. To investigate whether the small RNA sequences identified in small RNA libraries from rice leaves represent novel miRNAs from rice. To accomplish this specific objective, we applied the specific criteria required for the annotation of plant miRNAs, including experimental and computational analyses.

2. To investigate the possible contribution of candidate miRNAs (miR-64, miR-75, miR-96, miR-98 and miR-203) in rice immunity. Towards this end, we generated transgenic rice lines overexpressing each candidate miRNA which were molecularly and phenotypically characterized. The properties of resistance/susceptibility to infection by the rice blast fungus *Magnaporthe oryzae* were evaluated.

3. To explore the feasibility of using the CRISPR/Cas9 system for genome editing of *MIR* genes in rice. This CRISPR/Cas9 genome editing technique has been applied to the functional validation of the miR-75 candidate.

4. To determine whether miR858 is involved in plant immunity. This study was approached in Arabidopsis plants.

Results obtained in the identification and functional characterization of rice miRNAs (objectives 1, 2 and 3) are presented in Chapters I and II of this Thesis.

Results obtained on the study of miR858 in Arabidopsis (objective 4) are presented in Chapter III of this Thesis.

CHAPTER I

Identification and functional characterization of novel miRNAs from rice

Abstract

Plants defense responses against pathogens are mediated by the activation and repression of a large array of genes. Although many studies have focused on the transcriptional regulation of defense responses, less is known about the involvement of microRNAs (miRNAs) as post-transcriptional regulators of gene expression in plant immunity. Here, miRNAs were investigated for their functional role in the defense response of rice plants to infection by the fungal pathogen *Magnaporthe oryzae*, the causal agent of the rice blast disease. Based on the information gained by small RNA sequencing of rice tissues treated with *M. oryzae* elicitors, we identified 5 miRNA candidates, named as miR-64, miR-75, miR-96, miR-98 and miR-203. The accumulation of mature miRNA species in rice leaves, both miRNA-5p and miRNA-3p species, has been experimentally validated for all miRNA candidates. For miR-64, miR-75 and miR-96, a DCL3- and/or DCL4-dependence for accumulation of mature miRNA sequences was demonstrated, supporting that they represent novel miRNAs from rice. Transgenic rice plants overexpressing either miR-75 or miR-64 exhibited enhanced resistance to *M. oryzae*, a phenotype that was associated with a stronger activation of *PR1b* (*Pathogenesis-related 1b*) expression during pathogen infection. Contrary to this, increased susceptibility to *M. oryzae* infection and a weaker induction of *PR1b* expression was observed in rice plants overexpressing miR-96. Taken together, these findings provide evidence that miR-75 and miR-64 are positive regulators of rice immunity, whereas miR-96, miR-98 and miR-203 negatively regulate the rice response to pathogen infection. Our data establish a key role of miRNAs in modulating the plant response to pathogen infection which reinforce the relevance of miRNAs in rice innate immunity.

Introduction

Plants have evolved a complex and effective immune system that protect them from invading microorganisms. The first line of defense occurs through recognition of conserved Pathogen Associated Molecular Patterns (PAMPs) by host Pattern-Recognition Receptors (PRRs). Sensing PAMPs triggers a general defense response referred to as PAMP-triggered immunity (PTI), which operates against most pathogens (Jones and Dangl, 2006; Boller and Felix, 2009). Among others, PTI components include deposition of callose, production of reactive oxygen species (ROS), activation of protein phosphorylation/dephosphorylation processes and accumulation of *Pathogenesis-related proteins* (*PRs*). To counteract this innate defense, pathogens produce effectors that suppress PTI. In turn, many plants have evolved another layer of immunity in which Resistance (*R*) genes participate. This type of immunity is called Effector-Triggered Immunity (ETI) and relies on the specific recognition of microbial effectors (or host proteins modified by effectors) by proteins encoded by *R* genes. This recognition triggers a rapid and effective host defense response. The essential role of the phytohormones salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) in resistance to pathogens is also well established (Glazebrook, 2005; Robert-Seilaniantz et al., 2011; Denancé et al., 2013).

PTI and ETI responses to bacterial and fungal pathogens have been historically considered as protein-based defense mechanisms that are regulated at the transcriptional level, largely independent from the RNA-based mechanisms that typically operate in antiviral defense. There is, however, increasing evidence to support post-transcriptional regulation of immune responses triggered by pathogen infection in plants, and host endogenous small RNAs are nowadays recognized as important players in reprogramming gene expression in processes associated to PTI and ETI responses (Katiyar-Agarwal and Jin, 2010; Pumplin and Voinnet, 2013; Seo et al., 2013; Staiger et al., 2013; Gupta et al., 2014; Baldrich and San Segundo, 2016).

In plants, there are two main classes of small RNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are distinguished by their mode of biogenesis and mechanism of action (Chapman and Carrington, 2007; Vazquez and Hohn, 2013). Plant miRNAs are transcribed from *MIR* genes by RNA polymerase II as long single-stranded RNA precursors with unique stem-loop structures, the primary-miRNAs (or pri-miRNAs), which are processed in two steps by DICER-LIKE proteins (DCL, typically DCL1) to give

rise to double stranded miRNA duplexes (miRNA/miRNA*, also named miRNA-5p/miRNA-3p duplexes). Both strands of the miRNA/miRNA* duplex are methylated at the 3' end and transported to the cytoplasm where the functional miRNAs are loaded into the RNA-induced silencing complex (RISC). ARGONAUTE1 (AGO1) is the core component of the RISC complex (Rogers and Chen, 2013). MiRNAs guide post-transcriptional gene silencing through sequence-specific cleavage or translational repression of target mRNAs.

Plant miRNAs are known to regulate many developmental processes, including leaf, flower, root development, and hormone signalling (Palatnik et al., 2003; Mallory et al., 2004). Alterations in the accumulation of an important number of miRNAs are also observed during the plant response to environmental stresses, both abiotic and biotic stress (e.g. drought, cold, salinity, nutrient deficiency, pathogen infection) (Jagadeeswaran et al., 2009; Pumplin and Voinnet, 2013; Staiger et al., 2013; Seo et al., 2013; Campo et al., 2013; Li et al., 2014; Gupta et al., 2014; Baldrich and San Segundo, 2016). The first evidence for miRNAs affecting pathogen defense came from studies in *A. thaliana*, where a fragment of 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). Other examples of Arabidopsis miRNAs with a known function in disease resistance are: miR160a, miR393, miR398b, miR400, miR472, miR844 and miR863 (Navarro et al., 2006; Li et al., 2010; Boccara et al., 2014; Park et al., 2014; Lee et al., 2015; Niu et al., 2016). A direct role of miRNAs in controlling the expression of the major class of *R* genes, or the atypical ARLPK (*receptor-like pseudokinase*) gene, is also described (Niu et al., 2016). A priori, miRNAs can function as positive or negative regulators in PTI and ETI by targeting negative or positive defense regulators, respectively.

Even though an important number of plant miRNAs are known to be regulated by pathogen infection, our understanding of the functional roles of miRNAs in plant disease resistance is far less than that in plant development. Most research on miRNAs involved in plant immunity have been conducted in the model dicotyledoneous plant *Arabidopsis thaliana* infected with the bacterial pathogen *P. syringae,* or treated with flg22 from *P. syringae* (Navarro et al., 2006; Li et al., 2010; Boccara et al., 2014; Lee et al., 2015; Soto

et al., 2017). Less is known about miRNAs controlling resistance to infection by fungal pathogens in plants.

Most of the miRNAs that were initially identified are conserved throughout the plant kingdom and have conserved target genes. Conserved miRNAs are often highly expressed and target transcription factor genes involved in the regulation of diverse developmental processes (Jones-Rhoades et al., 2006). Plants also express evolutionarily non-conserved miRNAs that generally exists only in limited species and accumulate in a time and/or spatial-restricted manner, and are weakly expressed (Zhang et al., 2006; Jones-Rhoades, 2012; Nozawa et al., 2012). It is generally assumed that non-conserved miRNAs might function in specific biological processes and/or adaptation to environmental changes.

Rice (*Oryza sativa*) is the most widely consumed staple food for a large part of the world's human population. A broad range of diseases affects rice production, the rice blast disease caused by the fungus *Magnaporthe oryzae* being the most devastating fungal disease of cultivated rice worldwide. Rice improvement for durable resistance to blast based on *R* genes is difficult as most of the resistance genes break down in a few years because of the race specificity and the rapid change in pathogenicity of the blast fungus. At present, blast disease control relies on the repeated use of agrochemicals which has an adverse impact on human health and environment.

In recent years, a significant progress has been made in the identification of miRNAs from rice (Campo et al., 2013; Baldrich et al., 2015). There are examples of rice miRNAs whose function is crucial in controlling traits of agronomic importance, such as tiller growth, early flowering and grain production (Miura et al., 2010; Wang et al., 2012; Zhang et al., 2013). However, despite the notable number of miRNAs that are known to be regulated during *M. oryzae* infection, the exact role of these pathogen-responsive miRNAs in rice immunity remains elusive. Only for three rice miRNAs, a functional role in resistance to the blast fungus *M. oryzae* has been demonstrated. They are: miR160a, miR398b and miR7695 (Campo et al., 2013; Li et al., 2014). This fact reflects the important gap that occurs between identified miRNAs and our knowledge of the biological function of miRNAs in rice plants.

Owing to the scientific and economic importance of the rice/*M. oryzae* pathosystem, we are interested on the identification of rice miRNAs involved in the host defense response to *M. oryzae* infection. Towards this end, we described the small RNA population in rice

tissues that had been treated, or not, with *M. oryzae* elicitors (Campo et al., 2013; Baldrich et al., 2015). High-throughput sequencing of small RNA libraries from rice revealed that a high proportion of rice miRNAs are elicitor-responsive. In particular, we described a novel miRNA from rice, miR7695 that positively regulates resistance to infection by the rice blast fungus *M. oryzae* (Campo et al., 2013). Deep sequencing of small RNA populations from rice also allowed us to identify small RNA sequences representing novel miRNA candidates potentially involved in blast disease resistance. In this study, 5 novel miRNA candidates have been investigated for their role in disease resistance. Depending on the miRNA under study, its overexpression in stable transgenic plants confers resistance or susceptibility to infection by the rice blast fungus, indicating that they function as positive or negative regulators of rice immunity, respectively. Identifying novel miRNAs from rice will lay a comprehensive foundation for unraveling the complex miRNA-mediated processes that function in rice immunity.

Results

Our group previously characterized the small RNA population of rice tissues (leaves, roots) that have been treated, or not, with *M. oryzae* elicitors. Small RNA libraries were subjected to pyrosequencing (454 Life Sciences; Roche) (Campo et al., 2013). The small RNA sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30583). Small RNAs belonging to the known non-coding RNA families deposited in the Rfam Genbank database (rRNA, tRNAs, small nuclear RNAs and small nucleolar RNAs) were removed from small RNA datasets. For details about the bioinformatics analysis of sequencing data, see Campo et al (2013).

The small RNA sequences perfectly mapping to the rice genome *O. sativa*, cv Nipponbare version 7.0; http://rice.plantbiology.msu.edu/) were searched against the miRBase database (release 21, 2014) to identify known miRNAs (Campo et al., 2013). The remaining small RNA sequences mapping to the rice genome were further analyzed to identify novel miRNAs potentially involved in disease resistance in rice as follows.

1. Identifying novel miRNAs from rice.

Several criteria have been established for the identification and annotation of novel miRNAs which are based on both experimental and computational data (Ambros et al., 2003; Meyers et al., 2008). The specific criteria for the experimental verification of miRNAs consist of expression and biogenesis criteria. The biogenesis criteria include the excision form a stem-loop precursor transcript, whereas the expression criteria include the identification of the small RNA in plant tissues (e.g. detection by hybridization). Detection of small RNA sequences originating from opposite arms of the same precursor with a 2-nt 3' overhand, a signature of DCL activity (e.g. miRNA-5p and miRNA-3p species, previously named miRNA and miRNA* species) strengthens confidence in annotating novel small RNAs as miRNAs (Meyers et al., 2008). The expression criteria refers to the identification of the small RNA by cloning, small RNA sequencing, and/or detection of the small RNA in plant tissues via RNA hybridization (e.g. Northern blot analysis; *in situ* miRNA hybridization, etc).

Importantly, identification of novel miRNAs generally requires expression analysis in *dcl* mutants (Ambros et al., 2003). Canonical miRNAs are typically processed by DCL1, but certain miRNAs have been shown to require DCL3 or DCL4 activities for their production in Arabidopsis and rice plants (Rajagopalan et al., 2006, Wu et al., 2010; Campo et al., 2013). Then, *dcl1, dcl3* and *dcl4* mutants are useful tools for annotation of novel miRNAs in plants. Depending on the plant species under study, however, *dcl* mutants might not be available. In the absence of these genetic tools, annotation is supported by conservation in other species of the miRNA sequence and precursor secondary structure.

The initial selection of small RNA sequences of interest (designated as novel miRNA candidates) was based on the ability of flanking sequences to fold-back in a hairpin structure. For this, the rice genome was scanned for stem-loop hairpin structures comprising the small RNA sequences identified in the small RNA transcriptomes of rice leaves that did not match any known miRNA (i.e miRNA annotated in miRBase). For each small RNA sequence that had a perfect match in the rice genome, we determined the ability of the surrounding genomic sequences to fold into stem-loop precursor structures by using a maximum length of 3kb. Following the computational prediction of stem-loop structures, we selected 5 hairpin structures (hereinafter called novel miRNA candidates) for further analysis. The predicted hairpin structures of these candidates are presented in Figure 1.

Their names are hyphenated to distinguish them from annotated miRNAs. In all the miRNA candidates, the small RNA sequences identified in small RNA sequencing data sets mapped into the 5’ arm of the stem loop.

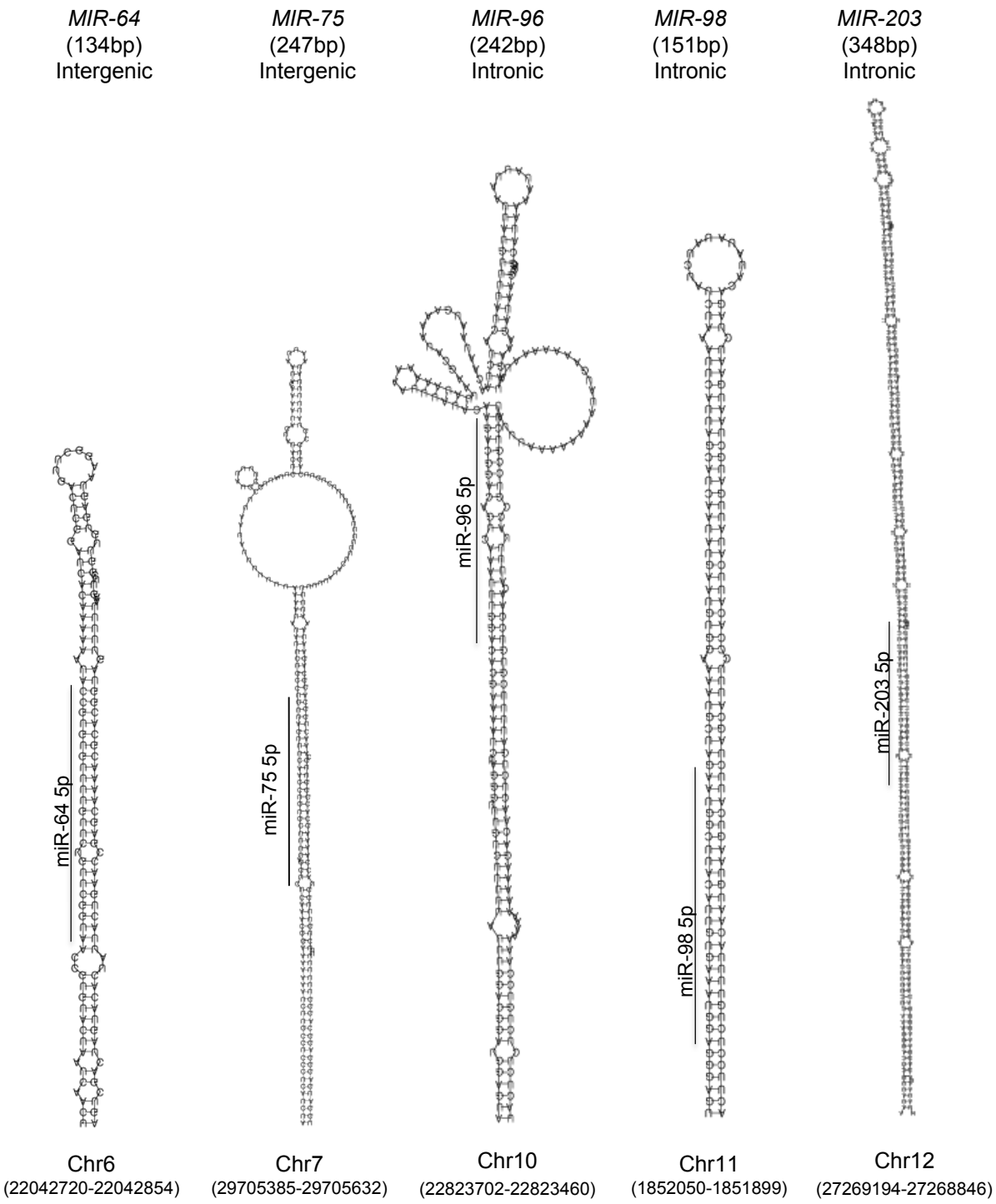


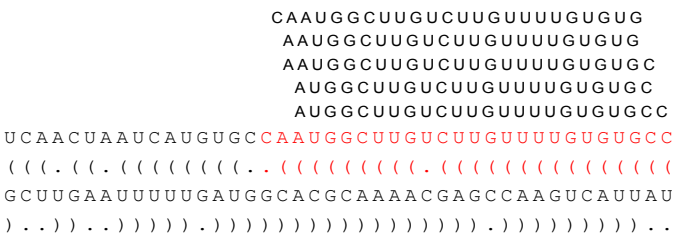
Figure 1. **Precursor structures and chromosomal location of novel miRNA candidates from rice**. Small RNA sequences mapping into these structures are represented by black bars (the suffix -5p refers to the mapping arm within the stem-loop). Additional information on their nucleotide sequences is presented in Figure 2.

Two of the five miRNA candidates (miR-64, miR-75) are located in intergenic regions, whereas the other three candidates (miR96, miR-98 and miR-203) mapped to the intronic region of a gene. The small RNA sequences recovered from the sequencing data mapping into these precursor structures are presented in Table 1, and the nucleotide sequences of the 5 precursor structures are shown in Figure 2. Alignment of sequencing reads mapping to the miR-64 precursor structure revealed the existence of variant forms for miR-64-5p, or isomiRs (Figure 2). IsomiRs are miRNA variants that are thought to be generated by alternate Dicer cutting, post-transcriptional RNA editing events, or by nucleotidyltransferases (Sablock et al., 2015).

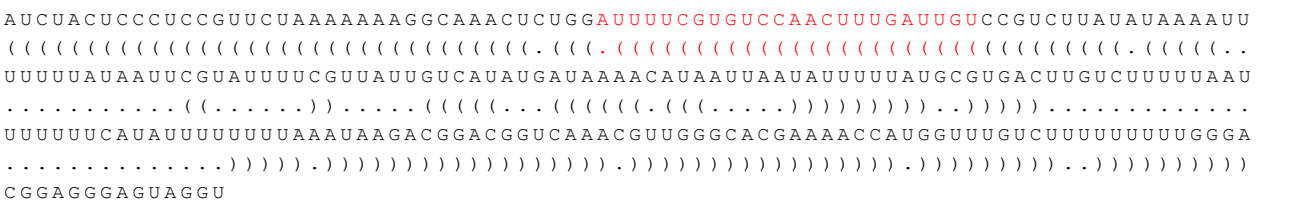
nucleotide sequence and chromosomal location of miRNAs candidates from rice				
			Organism	
precursor	**sequence**	**Chr**	**Monocot**	**Dicot**
miR-64	CAAUGGCUUGUCUUGUUUUGUGUG	6		*Tc*
miR-75	AUUUUCGUGUCCAACUUUGAUUGU	7		
miR-96	AAGACGGACAGUCAAAUUUGGA	10		
miR-98	GAAUGGCUUACAUUGUGAAAUGGA	11		
miR-203	UGAAAUAGAUCGCUAAUGUUGCAG	12	*El*	

Table 1. Nucleotide sequences of miRNA candidates from rice. The sequences given represent the small RNA sequences identified in small RNA libraries from rice tissues that have been treated, or not, with *M. oryzae* elicitors (Campo et al., 2013). The precursor sequence for each small RNA has the capacity to adopt hairpin structures in rice. A search for miRNA sequence homology was performed by BLASTN against National Center for Biotechnology Information (NCBI) genomes by allowing zero to three nucleotide substitutions. For those sequences mapping in the genome of any other species, the surrounding genomic sequences were analyzed to confirm their ability to form fold-back structures. *Tc*, *Theobroma cacao*; *El*, *Elaeis guineensis*.

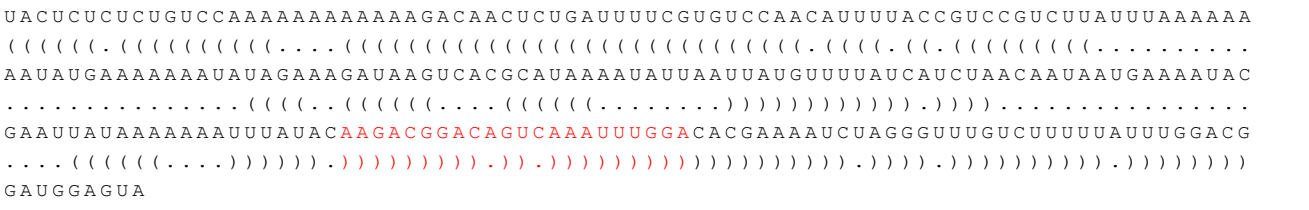
miR-64



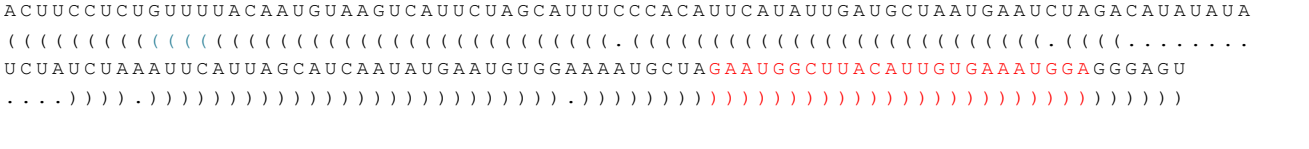
miR-75



miR-96



miR-98



miR-203

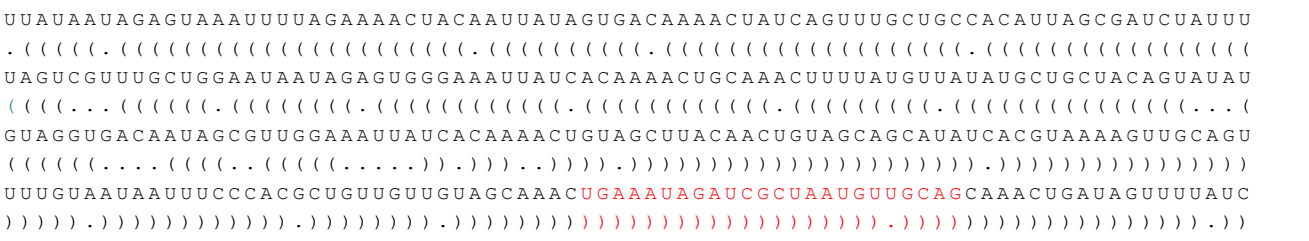


Figure 2. **Nucleotide sequences of the precursors containing novel miRNA candidates**. Small RNAs mapping at the 5p are indicated in red. The brackets represent base pairing and dots denote mismatches in the precursor sequences.

2. miR-75 and miR-64 function as positive regulators in resistance to the rice blast fungus *M. oryzae*.

2.1. Experimental validation of miR-75.

2.1.1. Detection of miR-75 in rice leaves.

miRNA validation relies on detection of the small RNA species in the plant tissue. Knowing that processing of a pre-miRNA by DCL activity gives rise to a miRNA duplex consisting of miRNA-5p and miRNA-3p strands (also known as miRNA/miRNA*), detection of small RNA sequences opposite to each other in a given miRNA precursor is used as a criterion for the identification of novel miRNAs.

Small RNA Northern blot analysis was carried out to validate miR-75 accumulation in rice tissues. As it is shown in Figure 3A, both miR-75-5p and miR-75-3p species were detected in rice leaves. Here, it is worthwhile to mention that a large amount of RNA was needed for miR-75 detection by Northern blot analysis (i.e., the small RNA fraction obtained from 500 µg of total RNA was analyzed) indicating that this small RNA accumulates at a low level in rice leaves.

Traditionally, the most abundant species of a miRNA/miRNA* duplex was assigned as the functional mature miRNA, and the miRNA* species was thought to be present at a low level. There are, however, many examples in the literature indicating that the abundance of miRNA* species relative to the mature miRNAs might vary widely. Thus, the sequence abundance might not always reflect the functional strand of the miRNA/miRNA* duplex (Rajagopalan et al., 2006). Evidence also support that the two small RNA species of a duplex can be functional, as illustrated by the Arabidopsis miR393 (Navarro et al., 2006; Zhan et al., 2011). As the functional strand of the mature miRNAs produced from the miRNA candidates under study in this work is not known, we will always refer to the small RNAs mapping to a given precursor structure as miRNA-5p or miRNA-3p species (for all the miRNA candidates under study).

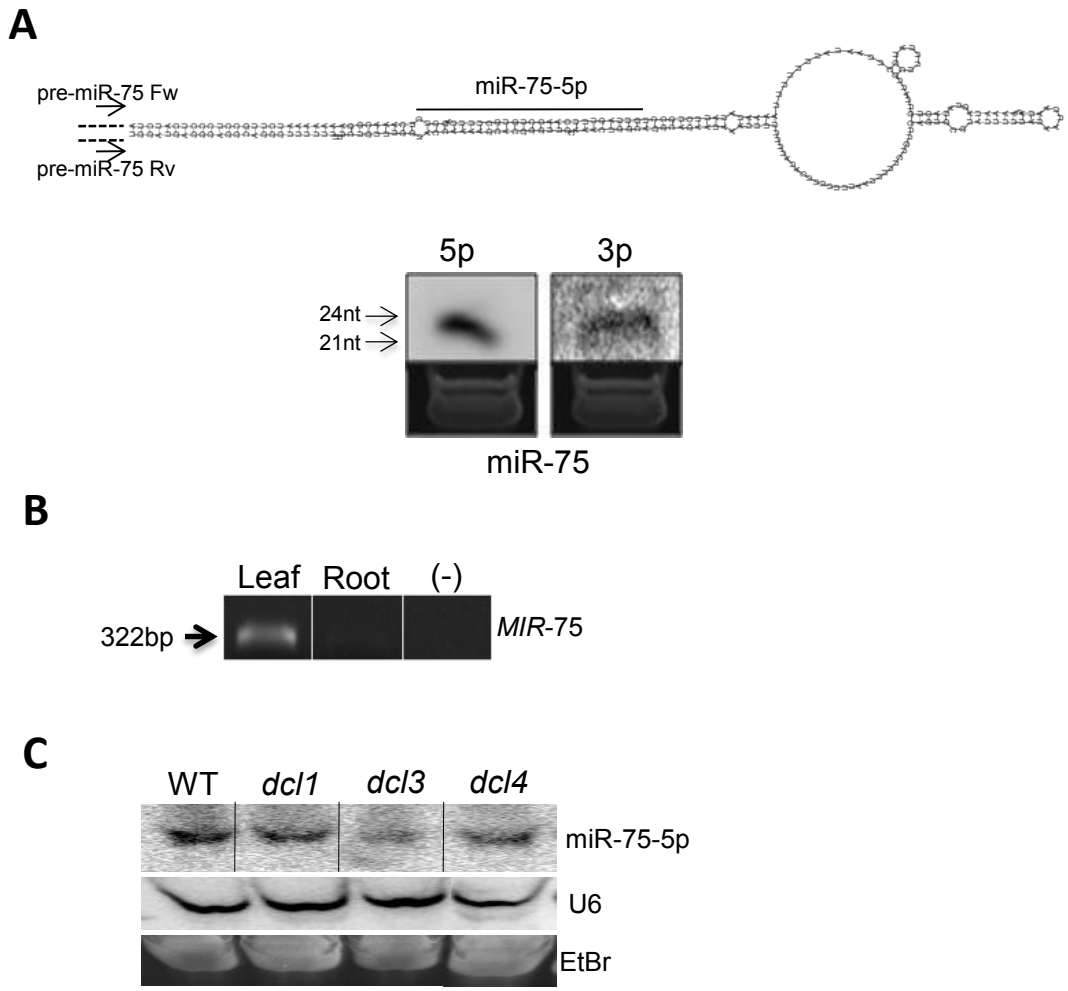


Figure 3. Experimental validation of miR-75. (A) Predicted hairpin structure of the miR-75 precursor structure. Small RNA sequences recovered from small RNA sequencing datasets (Campo et al., 2013) are represented by black bars. Arrows indicate the primers used for amplification in B. Northern blot analysis of small RNAs obtained from rice leaves, and corresponding ethidium bromide staining, is shown in the lower panel. Total RNA (300 µg) was used to obtain the small RNA fraction which was then were hybridized with a synthetic oligonucleotide probe, complementary to either miR-75-5p or miR-75-3p. **(B)** Detection of miR-75 precursor expression by RT-PCR. RNAs from leaves and roots of 21-old rice plants were analyzed. (-), RT-PCR reaction without reverse transcriptase. Sequencing of the PCR-amplified DNA fragment confirmed the specific amplification of the miR-75 precursor containing the expected small RNA sequences. **(C)** Northern blot analysis of miR-75-5p in rice mutants affected in miRNA biogenesis, the *dcl1*, *dcl3* and *dcl4* mutants. The same blot was stripped and re-probed with the U6 probe. The lower panel shows the ethidium bromide staining of RNA samples.

We examined the accumulation of miR-75 precursor transcripts in leaf and root tissues by RT-PCR using specific primers designed to amplify the fold-back structure of the precursor. Sequencing of the PCR products confirmed the specific amplification of the miR-75 precursor sequence. This analysis revealed that miR-75 precursor transcripts accumulate in leaves, but not in roots, of 3 week-old rice plants (Figure 3B). Most importantly, this result confirmed that the miR-75 precursor structure is transcribed as a

single transcriptional unit comprissing the two small RNA species detected by small RNA Northern blot. During miRNA biogenesis the pri-miRNAs are cleaved in the nucleus into shorter precursor miRNAs (pre-miRNA). As the cleavage of the pre-miR-75 sequence from the primary transcript (pri-miRNA) is unknown, it is not possible to know the exact sequence of the pre-miR-75 and pri-miR-75 transcripts. In this work, the predicted hairpin structures will be always referred to as miRNA precursors (or pre-miRNAs).Conservation among species is also used as a criterion for the assignment of a small RNA as a miRNA, which includes the finding of a conserved mature miRNA sequence coupled with a predictable secondary structure of the miRNA surrounding genomic sequence. Accordingly, a search for miRNA sequence homology was performed by BLASTN against National Center for Biotechnology Information (NCBI) genomes by allowing zero to three nucleotide substitutions. For those sequences mapping in the genome of any one plant species, the surrounding genomic sequences were analyzed to confirm their ability to form fold-back structures. miR-75 had no obvious orthologs in any other plant species for which genomic sequences are available (NCBI database) (Table 1).

2.1.2. Accumulation of miR-75 in *dcl* mutants.

Evolution of *MIR* genes is associated with gradual, overlapping changes in DCL usage from young to old *MIR* genes. It is generally assumed that the ancient, canonical 21-nucleotide miRNAs are generated by the activity of DCL1 (Bonnet et al., 2004; Rajagopalan et al., 2006; Vazquez et al., 2008), whereas recently evolved *MIR* genes are preferentially processed by DCL3 and DCL4 activities to produce different sizes of miRNAs (Rajagopalan et al., 2006; Vazquez et al., 2008; Axtell et al., 2011; Cuperus et al., 2011). Recently evolved *MIR* genes also have stem structures with few bulges.

In this work, we examined the accumulation of miR-75 in the *dcl1, dcl3,* and *dcl4* knockdown rice mutants (RNA interference mutants) (Liu et al., 2005, 2007; Song et al., 2012). Dr. X. Cao (Chinese Academy of Sciences, Beijing, China) kindly provided us with these mutants. As it is shown in Figure 3C, miR-75 accumulation was found to be severely compromised in the *dcl3* mutant, but not in the *dcl1* mutant, supporting that the miR-75 precursor is processed by DCL3. Whether miR-75 precursor processing is also dependent on DCL4 activities is not clear yet. This precursor also adopts a highly-complementary structure, a typical feature of recently evolved miRNAs. Thus, miR-75 most probably represents a novel, recently evolved miRNA from rice.

2.1.3. Production and molecular characterization of rice plants constitutively expressing miR-75.

To get further insights into the function of miR-75, we generated stable transgenic plants overexpressing miR-75. For this, the DNA fragment containing the predicted precursor sequence was amplified by PCR from rice genomic DNA (*O. sativa* cv *Nipponbare*), and cloned into the pCAMBIA1300 expression vector. Expression of the miR-75 precursor was driven by the *Ubiquitin 1* (*Ubi1*) promoter (Figure 4A).

Before rice transformation, we confirmed that the cloned genomic DNA fragment encompassing the miR-75 precursor was functional and produced the miR-75-related small RNA sequences. For this, agroinfiltration assays were carried out in *Nicotiana benthamiana* leaves. To avoid transgene-derived production of siRNAs, *the rdr6IR* line was used in these experiments (Schwach et al., 2005). As control, *N. benthamiana* leaves were agroinfiltrated with the empty vector (pCAMBIA1300). At 2 days after agroinfiltration, the leaf tissues were analyzed by Northern blot using oligonucleotide probes complementary to the miR-75-5p sequence. As it is shown in Figure 4B, mature miR-75 species accumulated in tobacco leaves that have been agroinfiltrated with the miR-75 precursor, which is absent in leaves agroinfiltrated with the empty vector. This finding demonstrated that the cloned miR-75 precursor is properly processed and produces the expected miR-75 sequence *in vivo*.

Transgenic rice lines were produced by *Agrobacterium*-mediated transformation using the *hptII* (*hygromycin phosphotransferase II*) gene encoding resistance to hygromycin as the selectable marker. As control, transgenic rice harbouring the empty vector (pCAMBIA1300) was also generated. Transgene integration and integrity into the rice genome was confirmed by PCR analysis of genomic DNA obtained from leaves of hygromycin-resistant lines (T0 plants; representative results are presented in Figure 4C). The accumulation of miR-75 small RNA sequences and precursor transcripts varied among the different transgenic lines (OE miR-75 lines), as revealed by small RNA Northern blot analysis and RT-qPCR, respectively. The accumulation level of mature and precursor miR-75 sequences of 3 representatives transgenic lines are shown in Figures 4D and E. Selected T0 plants were grown under greenhouse conditions to obtain homozygous transgenic lines (T2 generation). The transgene copy number was

estimated by qPCR in the T3 generation using the single copy *SPS* (*sucrose phosphate synthase*) gene for normalization (Table 2).

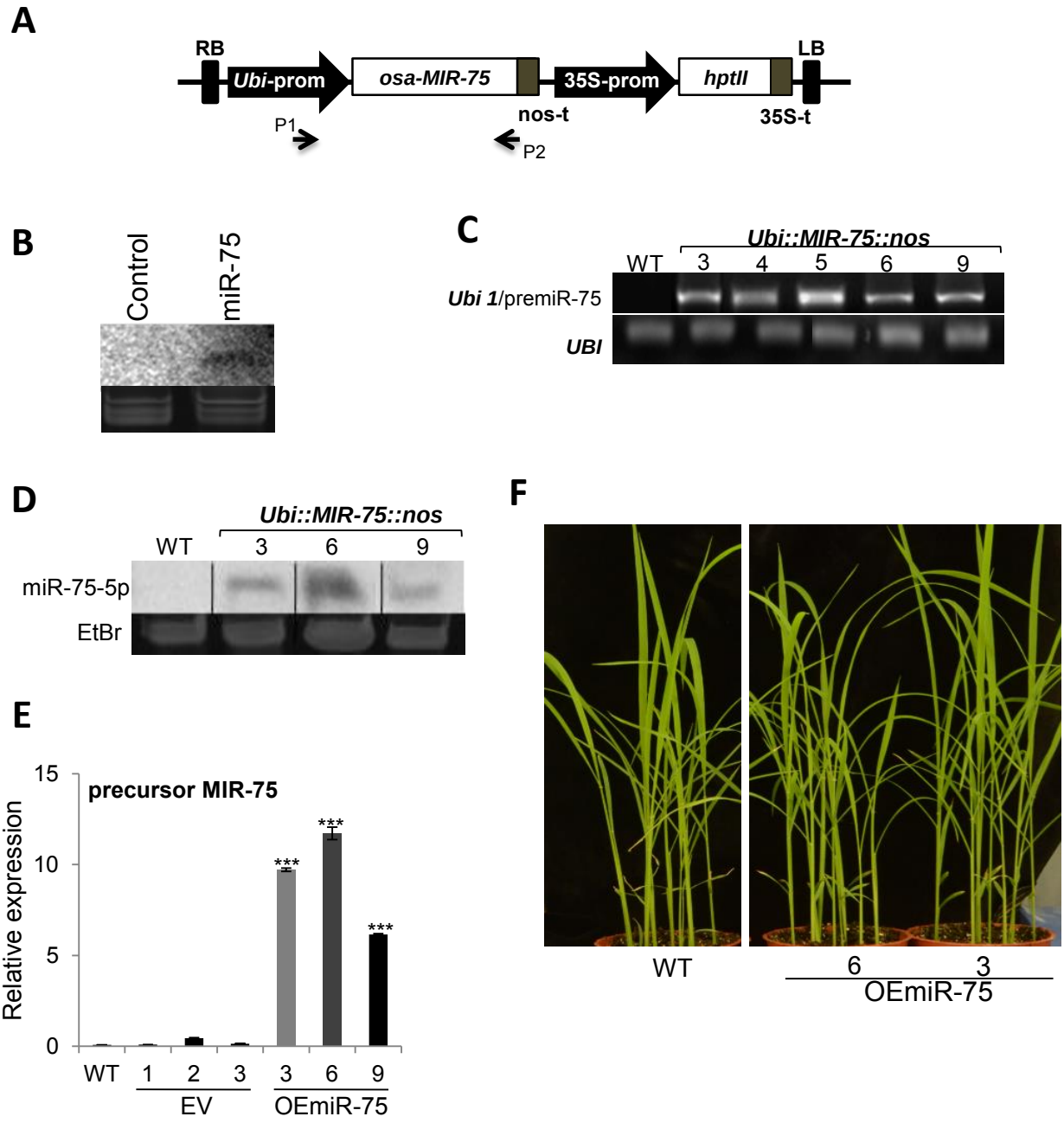


Figure 4. Molecular characterization of rice plants overexpressing miR-75. (A) Schematic diagram of the plant expression vector used for constitutive expression of miR-75 in rice. Expression of the miR-75 precursor is driven by the maize ubiquitin promoter (*Ubi-prom*) and the *nopaline synthase* terminator (*nos-t*). The *hptII* (*hygromycin phosphotransferase*) gene encoding resistance to hygromycin serves as a selectable marker for rice transformation. *35S-prom*, 35S promoter from the cauliflower mosaic virus. RB, right border; LB, left border. Arrows indicate the primers used for PCR amplification in C. **(B)** Functional analysis of the miR-75 precursor in *N. benthamiana* leaves (*rdr6IR* line). The miR-75-5p sequence produced from this precursor was detected by agroinfiltration of *N. benthamiana* leaves, followed by northern blot analysis using oligonucleotides complementary to miR-75-5p. No signal was detected in control leaves agroinfiltrated with the empty pCAMBIA1300 vector (control). **(C)** Transgene integration and integrity was confirmed by PCR analysis of genomic DNA of independent transgenic lines. Primers used for PCR located at the 3' end of the *Ubiquitin* promoter (*Ubi1*) and miR-75 precursor (downstream of the

sequence forming the stem-loop structure). As a negative control WT (wild-type) plants were used **(D)** Northern blot analysis of miR-75-5p in leaves of independent transgenic rice lines overexpressing miR-75. Results obtained for representative transgenic lines with different levels of miR-75 accumulation are shown. **(E)** Accumulation of pre-miR-75 transcripts in leaves of plants determined by RT-PCR**.** WT (wild-type), EV (transgenic rice lines harbouring the empty vector). Statistical significance was determined by ANOVA (***, P ≤ 0.001). The histogram shows the mean ± SD. **(F)** Appearance of 3 week-old miR-75 and wild-type plants grown under controlled greenhouse conditions.

Three independently generated transgenic lines harboring a single copy of the transgene were further analysed (lines 3, 6 and 9). Under normal growth conditions, there were no obvious phenotypical differences between homozygous transgenic lines constitutively expressing miR-75 (hereinafter OE miR-75 lines) and wild-type plants (Figure 4F).

	Lines	Number of copies
OEmiR-75	3	1,06
	6	0,99
	9	1,13
EV	1	0,96
	2	0,99
	3	1,07

Table 2. Determination of transgene copy number in rice lines overexpressing miR-75 by qPCR using the *sucrose phosphate synthase* (*SPS*) gene as the endogenous reference gene (Yang et al., 2004).

2.1.4. Constitutive expression of miR-75 confers enhanced resistance to *M. oryzae* infection.

To examine the functional relevance of miR-75 overexpression in rice, the OEmiR-75 lines were assayed for resistance to infection by the rice blast fungus *M. oryzae*. Three independent OE miR-75 lines at the 3-leaf stage were spray-inoculated with a spore suspension of *M. oryzae* ($5x10^6$spores/ml). As controls, transgenic lines overexpressing the empty vector and wild-type plants were used. In these experiments, azygous plants (progeny of transgenic parent lines that have lost the transgene through segregation) were also included in infection experiments. Compared with control plants, the OEmiR-75 lines consistently exhibited resistance to *M. oryzae* infection (Figure 5A).

Image analysis was used to determine the percentage of leaf area affected by blast lesions. In agreement with the visual inspection, leaves of the fungal-infected OE miR-75 plants showed a lower percentage of diseased area relative to leaves of the fungal-infected control plants (Figure 5B). Depending on the line, leaves from OE miR-75 plants exhibited 3-5% of their area affected by blast lesions at 7 days post-inoculation whereas, under the same experimental conditions, leaves of control plants were affected in 40-

43% of their area. Resistance to *M. oryzae* in OE miR-75 plants was further confirmed by quantifying fungal DNA in the infected leaves by qPCR (Figure 5C). From these results, it is concluded that miR-75 overexpression confers enhanced resistance to infection by the fungal pathogen *M. oryzae*.

Resistant phenotypes are usually accompanied by the up-regulation of defense-related genes, and the expression of *PR* (*Pathogenesis-Related*) genes is a widely used indicator of induction of plant defense responses. In particular, induction of *PR1* expression is considered a marker of the activation of defense responses to *M. oryzae* infection (Agrawal et al., 2001).

Having established that miR-75 overexpression confers resistance to *M. oryzae* infection, we examined the expression of *PR1b,* a member of the *PR1* family of *PR* (Pathogenesis-Related) genes, in mock-inoculated and *M. oryzae*-inoculated miR-75 and wild-type plants. As expected, induction of *PR1b* expression was observed in empty vector control plants at 24 and 48h post-inoculation (Figure 5D). Interestingly, a higher induction of *PR1b* expression occurred in OE miR-75 plants compared to that in control plants, indicating that the resistance phenotype that is observed in OE miR-75 plants might be, at least in part, due to a stronger activation of defense responses during pathogen infection.

2.1.1. Predicted targets of miR-75.

Understanding the biological function of miRNAs requires the identification of their target genes. Prediction of plant miRNA targets has been facilitated by the extensive sequence complementarity between plant miRNAs and their targets, and several miRNA target prediction programs have been developed. In higher plants, most miRNAs function by cleaving their corresponding targets, and cleavage normally occurs at the 10-11 nucleotide positions from the 5’ end of the miRNA (Llave et al., 2002; Jones-Rhoades et al., 2006; Rhoades et al., 2002).

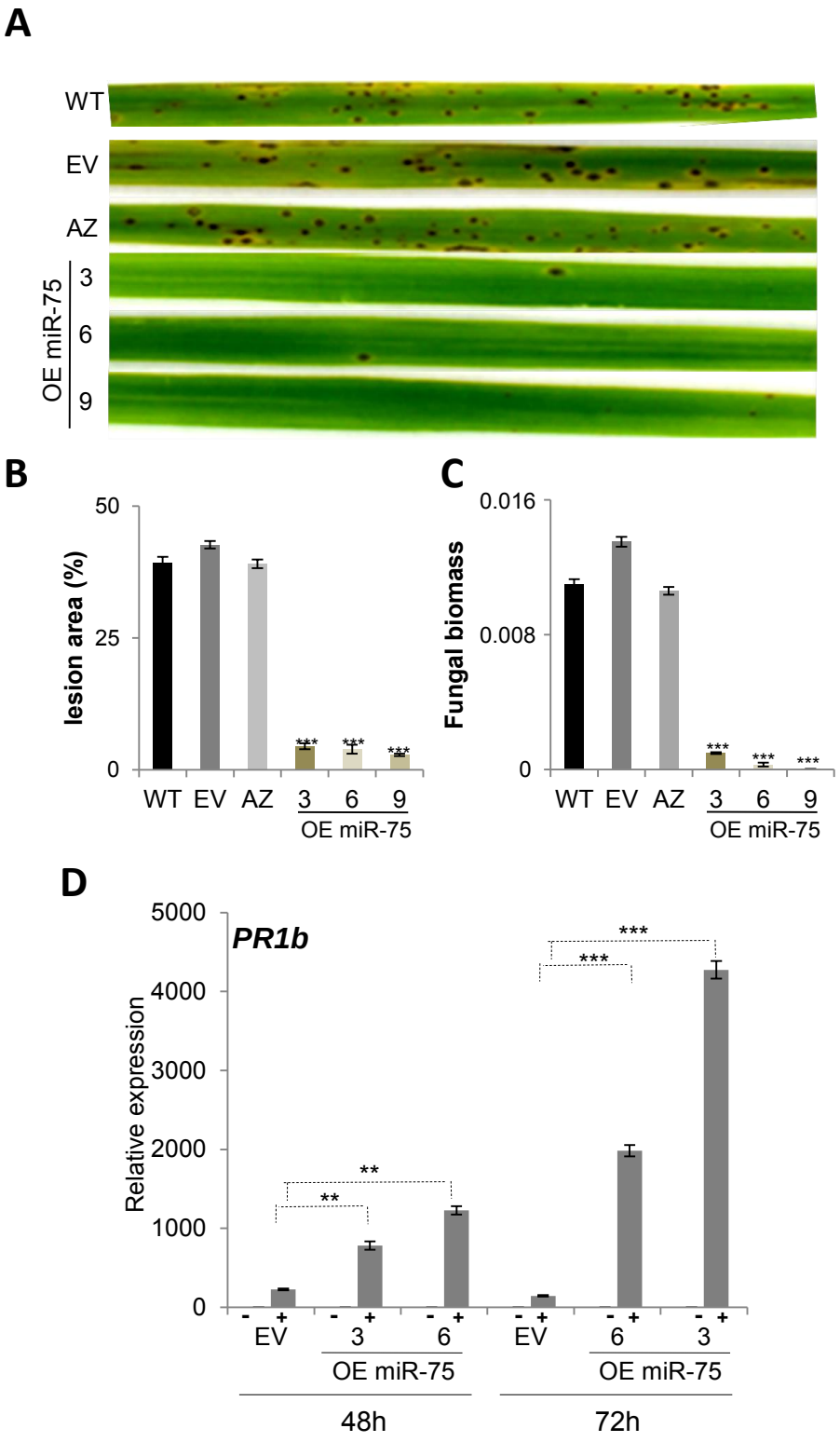


Figure 5. Resistance of rice plants overexpressing miR-75 to infection by the rice blast fungus *Magnaphorte oryzae*. (A) Resistance was tested in three independent transgenic miR-75 (homozygous, T3 generation), empty vector (EV, three independent homozygous lines), and wild-type (WT, *Nipponbare)*; AZ (azygous controls segregated from the T0) plants. Plants at the three-leaf stage (15 plants/genotype) were inoculated with *M. oryzae* spores ($5x10^6$ spores/ml). At 7 days post-inoculation, disease symptoms at the second leaf were evaluated. Results are from one out of three independent infection experiments which gave similar results. **(B)** Percentage of the leaf area showing blast lesions (7dpi). **(C)**. Quantification of *M. oryzae* DNA by qPCR at 3 days post-inoculation using specific primers of the *M. oryzae 28S* gene. Values

are fungal DNA levels were normalized against the rice *UBIQUITIN* gene. Expression of defense marker gene *PR1b* in OEpremiR-96 plants and wild-type plants. Three-week old OE premiR-96plants were inoculated with *Magnaphorte oryzae spores*. **(D)** Expression analyses were carried out by RT-qPCR 48 and 72 hours after inoculation. Mock-inoculated and inoculated plants are represented by black and grey bars, respectively. As expected defense marker genes have a high respond to infection in transgenic lines plants compared to control plants. Statistical significance was determined by ANOVA (**, P ≤0.01; ***, P ≤0.001). Histograms show the mean ± SD.

To identify potential target genes of miR-75, we used the psRNAtarget server for plant miRNA target analysis (http://plantgrn.noble.org/psRNATarget/; Dai and Zhao, 2011). A stringent criterion for target prediction, i.e. blast searches for hits with no more than 3 mismatches, was applied to reduce the ratio of false positive predictions. On this basis, 2 feasible targets were predicted for miR-75-5p, whereas 8 targets were predicted for miR-75-3p (all of them with a score of ≤ 3.0) (Table 3).

We also searched for miR-75 target gene(s) in degradome libraries of rice tissues treated, or not, with elicitors obtained from *M. oryzae* (Baldrich et al., 2015); the same biological material used for preparation of small RNA libraries was used for preparation of degradome libraries). Degradome tags were identified for *glucan endo-1,3-beta-glucosidase 7* (*Os03g12620*), or β-1,3-glucanase (Table 3). The miR-75 target site locates at the 3' UTR region of β-1,3-glucanase transcripts, the two miRNA species of miR-75 (i.e. miR-75-5p and miR-75-3p) having complementary sites in these transcripts (Figure 6). Thus, detection of miR-75-mediated cleavage of β-1,3-glucanase transcripts supports a regulation of this gene by miR-75. In this respect, β-1,3-glucanases which are classified as the *PR2* family of *PR* proteins (van Loon et al., 1999), are hydrolytic enzymes with an important role in defense responses against pathogen infection. These enzymes degrade β-1,3-glucan, a major structural component of the cell wall of many pathogenic fungi. Future experimental validation will determine if any one of the other predicted targets is a real target for miR-75.

Predicted target genes for miR-75						
miRNA	**Predicted target**	**Locus ID**	**Score**	**Alignment**	**Cleavage position**	**Degradome analysis**
miR-75 (5p)	Glucan endo-1,3-beta-glucosidase 7	Os03g12620	0.5	||||||||||||||o|||	1762	Validated
miR-75 (5p)	Na+/H+ antiporter	Os09g02214	2.5	|||o||||||||o||||||	3105-3128	NO
miR-75 (3p)	Expressed protein	Os09g32940	1.0	|||| |||o|||||||||||	1521-1545	NO
miR-75 (3p)	CBL-interacting serine/threonine-protein kinase 15	Os03g22050	1.0	| ||o|||o||||||||||	2906-2930	NO
miR-75 (3p)	Expressed protein	Os11g09260	2.0	| |||||o|o||||||||||	890-914	NO
miR-75 (3p)	Expressed protein	Os10g10170	2.0	|||| | ||o|||||||||||	2806-2830	NO
miR-75 (3p)	Glucan endo-1,3-beta-glucosidase 7	Os03g12620	2.0	||| ||||o|o||||||||||	1482-1506	NO
miR-75 (3p)	Expressed protein	Os03g24730	2.5	||| ||||o||||||||||o||	81-105	NO
miR-75 (3p)	Eexpressed protein	Os03g48380	2.5	|||| ||||o|||||||| |||||	1592-1616	NO
miR-75 (3p)	Protein synthesis inhibitor I	Os01g06740	2.5	|| | ||||o||||||||||o||	1191-1215	NO

Table 3. Predicted target genes for miR-75 and validation by degradome analysis. Data of targets validated by degradome analysis were taken from Baldrich et al. (2015). Target prediction was perfomed with the psRNAtarget program. The circles indicate G::U pairing.

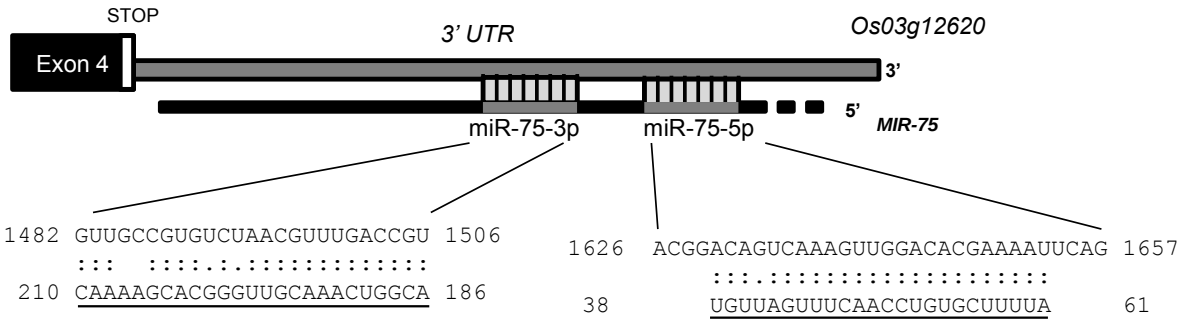


Figure 6. miR-75 targets the 3' untranslated (3' UTR) región of the *glucan endo-1,3-β-glucosidase 7* gene (β-1,3-glucanase), a member of the *PR2* family of *PR* proteins. The complementarity of miR-75-related small RNAs with the 3' UTR region of *Os03g12620* transcripts is shown. The cleavage site for miR-75-5p has been validated by degradome analysis (Baldrich et al., 2015).

Collectively, this study revealed that miR-75, is a novel miRNA from rice whose production is largely dependent on DCL3 activities. This finding, together with the long extensive base pairing within the stem region of miR-75 supports that this miRNA might be an evolutionarily recent *MIR* gene. MiR-75 accumulates in leaves, the host tissue for *M. oryzae* infection. Most importantly, overexpression of miR-75 enhances resistance to infection by the rice blast fungus *M. oryzae*, supporting that this miRNA functions as a positive regulator in rice immunity by modulating the expression of β-1,3-glucanase transcripts. Official naming of miR-75 awaits assignment by the miRBase repository.

2.2. Experimental validation of miR-64.

The same steps shown in Section 2.1 for the molecular and phenotypical characterization of rice plants constitutively expressing miR-75 were followed for the characterization and functional validation of miR-64. The results were as follows:

2.2.1. Detection of miR-64 in rice leaves.

The precursor structure and small RNAs sequences identified in small RNA libraries from rice are presented in Figure 7A. Northern blot analyses confirmed that the two miR-64 species, miR-64-5p and miR-64-3p, accumulate in rice leaves (Figure 7A). As it was found for miR-75, the miR-64 species accumulate at a low level in rice leaves (the small RNA fraction obtained from 300 µg of total RNA was analysed by small RNA Northern blot). The miR-75 precursor transcripts encompassing the miR-64-5p and miR-64-3p secuenced small RNAs were detected in leaves, but not in roots, of rice plants (Figure

7B). A search in the genome sequences available (NCBI database) revealed the presence of miR-64 in the genome of *Theobroma cacao* but not in any other monocotyledonous or dicotyledonous species (see Table 1).

2.2.2. Accumulation of miR-64 in *dcl* mutants.

When examining the accumulation of miR-64 in the three genetic backgrounds (*dcl1*, *dcl3* and *dcl4*), miR-64 accumulation was found to be significantly reduced in the *dcl3* and *dcl4* mutants Figure 7C. This observation suggests that miR-64 is processed by DCL3 and DCL4, a typical feature of recently evolved miRNAs.

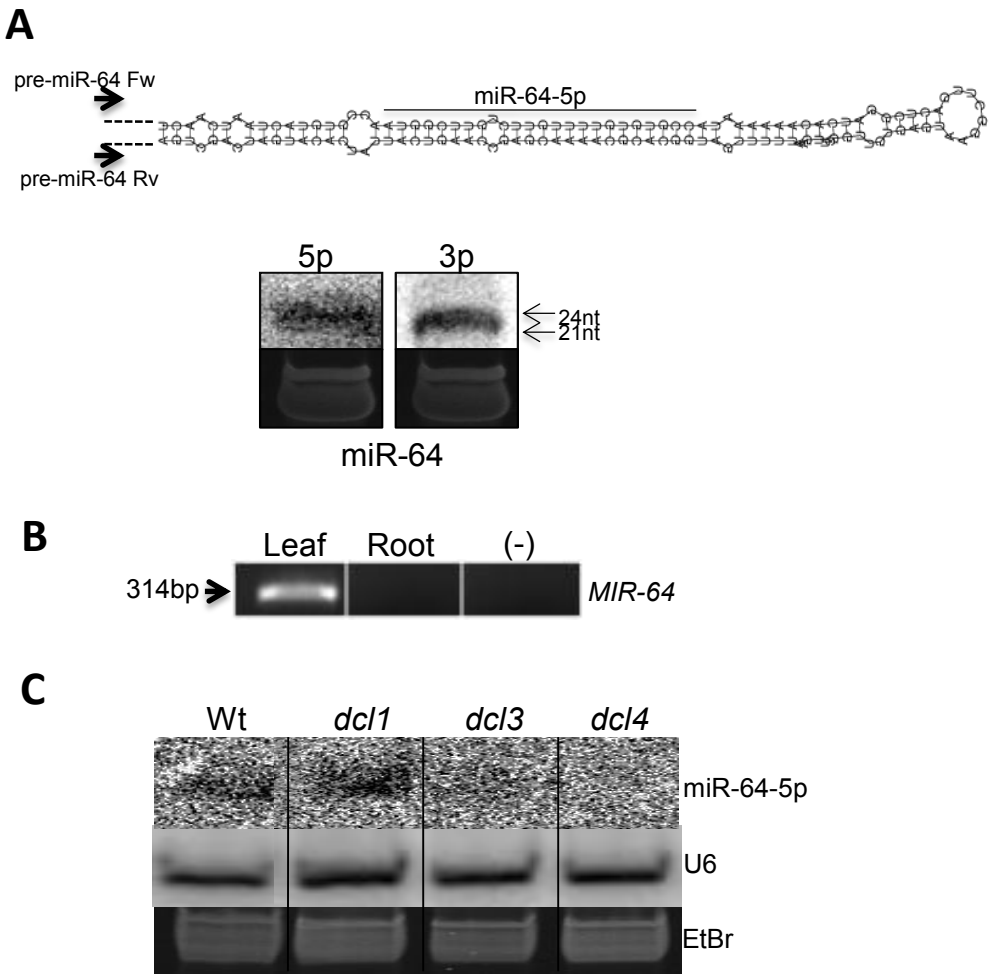


Figure 7. Experimental validation of miR-64. (A) Precursor structure of miR-64. Small RNA sequences recovered from small RNA sequencing datasets (Campo et al., 2013) are represented by black bars. Arrows (Fw, Rv) indicate the primers used for amplification in B. Northern blot analysis of small RNAs obtained from rice leaves, and corresponding ethidium bromide staining, is shown in the lower panel. Total RNA (500 µg) was used to obtain the small RNA fraction, which was then hybridized with a synthetic oligonucleotide probe complementary to the miR-64 sequences (-5p or -3p). **(B)** Detection of miR-64 precursor transcripts by RT-PCR. RNAs from leaves and roots of 21-old rice plants were analyzed. (-), RT-PCR reaction without reverse transcriptase. Sequencing of the PCR-amplified DNA fragment

encompassing the miR-64 precursor sequence confirmed the specific amplification of this precursor. **(C)** Northern blot analysis of miR-64-5p in rice mutants affected in miRNA biogenesis, the *dcl1*, *dcl3* and *dcl4* mutants. The same blot was stripped and re-probed with the U6 probe. The lower panel shows the ethidium bromide staining of RNA samples.

2.2.3. Production and molecular characterization of rice plants overexpressing miR-64.

Transgenic rice (*O. sativa* cv Nipponbare) lines constitutively expressing the miR-64 precursor sequence were generated. For this, the DNA fragment containing the stem-loop precursor structure of miR-64 was PCR-amplified from genomic DNA (Figure 8A), and cloned into the pCAMBIA1300 expression vector. Agroinfiltration experiments in *N. benthamiana* (*rdr6IR* line) leaves revealed accumulation of miR-64-5p sequences in tissues that have been agroinfiltrated with the miR-64 precursor, but not in leaves that have been transformed with the empty vector construct (Figure 8B). Thus, the cloned miR-64 precursor was functional and produced the expected miR-64 small RNA sequences *in vivo*.

Transgenic rice overexpressing miR-64 were produced by *Agrobacterium*-mediated transformation, and molecularly characterized as described in previous sections. Briefly, transgene integration and integrity (Figure 8C) was confirmed in the independently-generated T0 plants. Transgene expression was demonstrated by small RNA Northern blotting for miR-64-5p accumulation, and RT-qPCR for miR-64 precursor accumulation (Figure 8D, E). Stable *inheritance* and transgene expression was followed through successive *generations (up to th*e T3 generation). Transgenic rice plants showed normal growth and development (Figure 8F). Independently generated transgenic lines harbouring a single copy of the transgene were identified in the T3 generation (Table 4). Three independent transgenic lines were selected for further studies (lines 3, 5 and 7).

	Lines	Number of copies
OEmiR-64	3	1,05
	5	1,02
	7	1,02
EV	1	0,96
	2	0,99
	3	1,07

Table 4. Determination of transgene copy number in rice lines overexpressing miR-64 by qPCR using the *sucrose phosphate synthase* (*SPS*) gene as the endogenous reference gene (Yang et al., 2004).

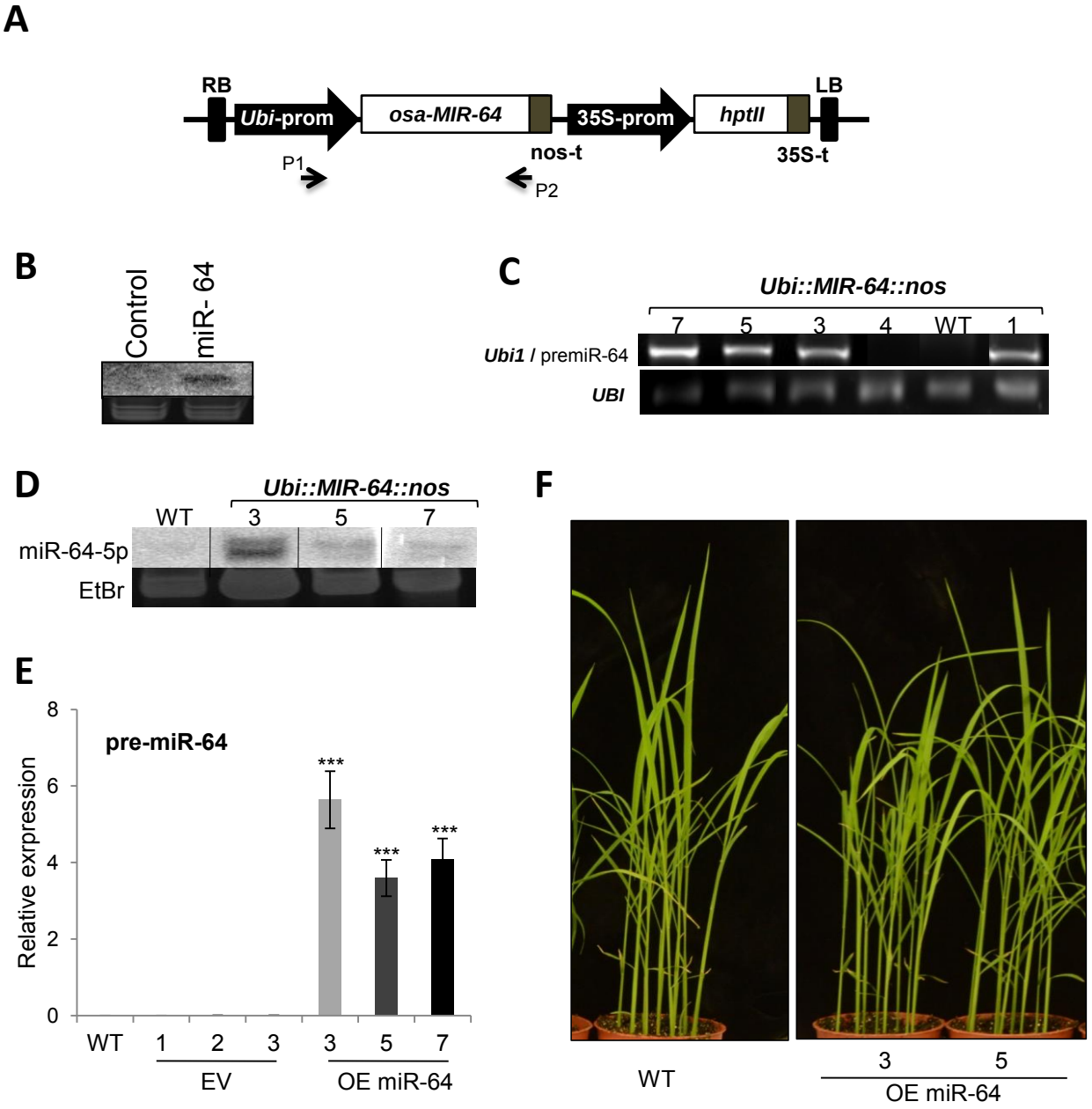


Figure 8. Molecular characterization of rice plants overexpressing miR-64. (A) Schematic diagram of the plant expression vector used for constitutive expression of miR-64 in rice. Expression of the miR-64 precursor is driven by the maize ubiquitin promoter (*Ubi-prom*) and the *nopaline synthase* terminator (*nos-t*). The *hptII* (*hygromycin phosphotransferase*) gene encoding resistance to hygromycin serves as a selectable marker for rice transformation. *35S-prom*, 35S promoter from the cauliflower mosaic virus. RB, right border; LB, left border. Arrows indicate the primers used for PCR amplification in C. **(B)** Detection of miR-64-5p sequences produced from the miR-64 precursor by agroinfiltration of *N. benthamiana* leaves (*rdr6IR* line), followed by northern blot analysis. No signal was detected in control leaves agroinfiltrated with the empty pCAMBIA vector (control). **(C)** Transgene integration and integrity in transgenic rice lines. PCR analysis of genomic DNA of independent transgenic lines using primers used for PCR located at the 3' end of the *Ubiquitin* promoter (*Ubi1*) and miR-64 precursor (downstream of the sequence forming the stem-loop structure). As a negative control WT plants were used **(D)** Northern blot analysis of miR-64-5p in leaves of independent transgenic rice lines overexpressing miR-64 plants. Results obtained for three representative transgenic lines with different levels of miR-64 accumulation are shown. **(E)** Accumulation of miR-64 precursor transcripts in leaves of plants determined by RT-qPCR. WT (wild-type), EV (empty vector). Statistical significance was determined by ANOVA (***, $P \leq 0.001$). The histogram shows the mean

± SD. **(F)** Appearance of 3 week-old miR-64 and wild-type plants grown under controlled greenhouse conditions.

2.2.4. Resistance to infection by the rice blast fungus *M. oryzae* in transgenic rice constitutively expressing miR-64.

The functional relevance of transgenic expression of miR-64 in resistance to infection by the rice blast fungus *M. oryzae* was examined. Three independent OE miR-64 lines at the 3-leaf stage and control plants (azygous, wild-type plants and transgenic plants harbouring the empty vector) were spray-inoculated with a spore suspension of *M. oryzae* ($5x10^6$spores/ml). As it is shown in Figure 9A, blast lesions developed in leaves of control plants but not in leaves of OE miR-64 plants. The ability of OE miR-64 plants to block the in planta growth of *M. oryzae* was further assessed by determining the percentage of lesion area and by quantification of fungal biomass in infected leaves of both control and OE miR-64 lines (Figure 9B, C). Finally, an induction of *PR1b* gene expression was observed in leaves of *M. oryzae*-infected OEmiR-64 and empty vector control plants. However, the OE miR-64 plants accumulate higher levels of *OsPR1b* transcripts than control plants at the two time points of infection here assayed (Figure 9D). Together, these studies demonstrated that constitutive expression of miR-64 in rice confers enhanced resistance to infection by the fungus *M. oryzae*, and that the phenotype of disease resistance is accompanied by a higher induction of *PR1b* expression. Thus, miR-64 appears to function as a positive regulator of rice immunity.

2.2.5. Predicted targets of miR-64.

By using the psRNAtarget program (http://plantgrn.noble.org/psRNATarget/), miR-64-5p was predicted to target the disease resistance *RPS2* gene (Table 5). In Arabidopsis, the *RPS2* protein specifically recognizes the AvrRpt2 type III effector avirulence protein from *Pseudomonas syringae* and triggers defense responses (Mackey et al., 2003). However, analysis of the degradome data from rice leaves (Baldrich et al., 2015) did not revealed cleavage of RPS2 transcripts, and accordingly, a miR-64-mediated cleavage of this gene could not be demonstrated in this work.

Predicted target genes for miR-64						
miRNA	**Predicted target**	**Locus ID**	**Score**	**Alignment**	**Cleavage position**	**Degradome analysis**
miR-64 (5p)	Disease resistance protein RPS2	Os09g14010	3.0	| o|o|o||||||||||||	185-208	NO
miR-64 (3p)	Retrotransposon protein, Ty3-gypsy subclass	Os08g11060	2.0	|| o|| ||||||||||||	127-152	NO
miR-64 (3p)	Hypothetical protein	Os05g27290	2.0	o|| || ||||||||||||	106-127	NO

Table 5. Predicted target genes for miR-64. Target prediction was carried out using the psRNAtarget program. Circles indicate G::U pairing.

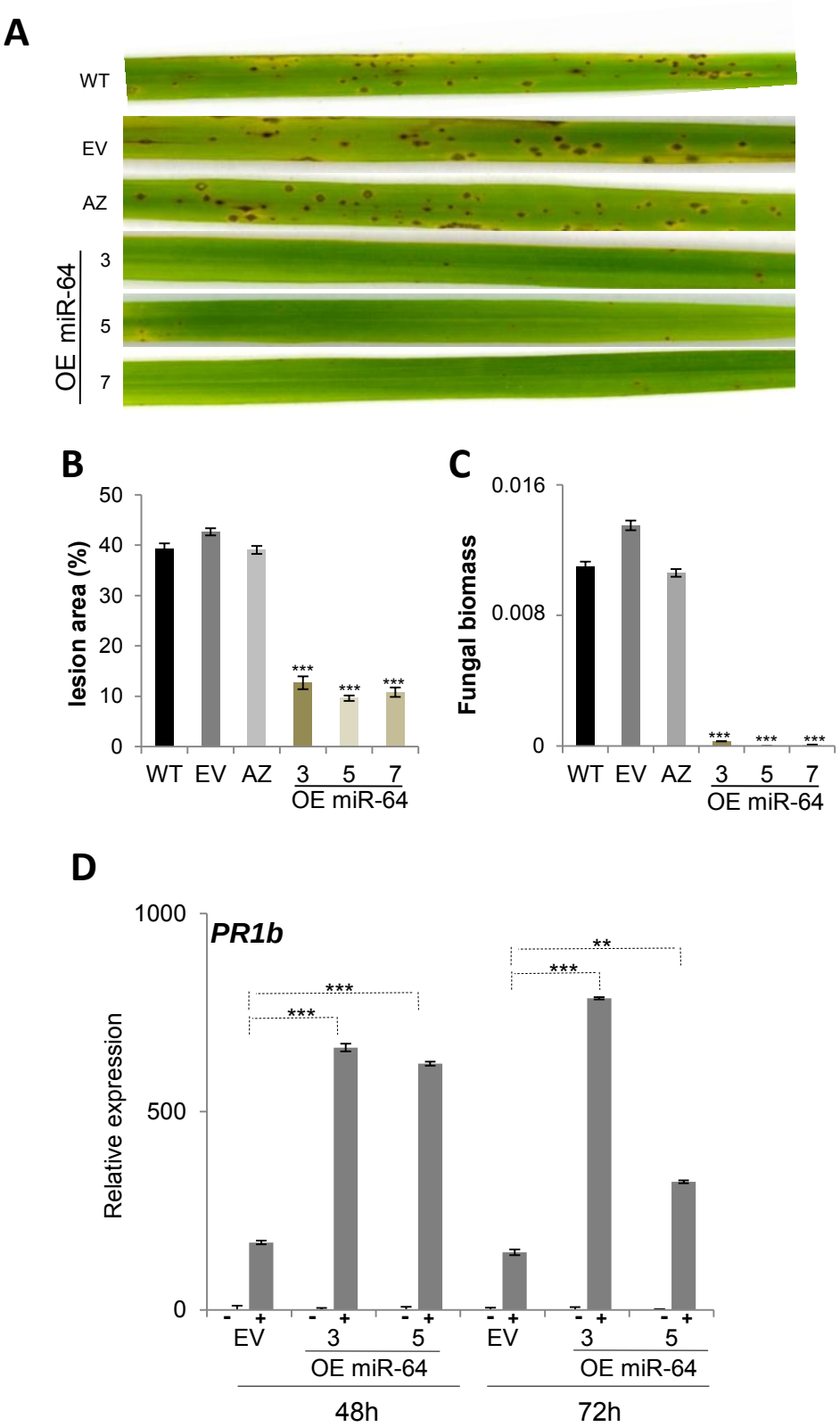


Figure 9. Resistance of rice plants constitutively expressing miR-64 to infection by the rice blast fungus *Magnaphorte oryzae*. (A) Resistance was tested in three independent transgenic miR-64 (homozygous, T3 generation), empty vector (EV, three independent homozygous lines), and wild-type (WT, *Nipponbare*; AZ, azygous controls segregated from the T0) plants. Plants at the three-leaf stage (15 plants/genotype) were inoculated with *M. oryzae* spores ($5x10^6$ spores/ml). Disease symptoms were evaluated in the second leaf of infected plants at 7 days post-inoculation. Results are from one out of three

independent infection experiments which gave similar results. **(B)** Percentage of the leaf area showing blast lesions (7dpi). **(C)**. Quantification of *M. oryzae* DNA by qPCR at 3dpi using specific primers of the *M. oryzae 28S* gene (Mo28S), and the *UBIQUITIN1* (*Os06g46770*) gene as the internal control. **(D)** Expression analyses were carried out by RT-qPCR 48 and 72 hours after inoculation. Mock-inoculated and inoculated plants are represented by black and grey bars, respectively. As expected defense marker genes have a high respond to infection in transgenic lines plants compared to control plants. Statistical significance was determined by ANOVA (**, P ≤0.01; ***, P ≤0.001). Histograms show the mean ± SD.

3. miR-96, miR-98 and miR-203 function as negative regulators in resistance to infection by the rice blast fungus *M. oryzae*.

3.1. Experimental validation of miR-96

3.1.1. Detection of miR-96 expression in rice leaves.

Two small RNA species derived from the opposite strands of the stem-loop structure of this precursor were found to accumulate in rice leaves, as revealed by small RNA Northern blot analysis (Figure 10A, B). The miR-96 small RNAs also accumulated at low levels (the small RNA fraction obtained to 200 ug of total RNA was needed for their detection). MiR-96 precursor transcripts were detected in the rice leaves, but not in roots of rice plants (Figure 10B). A search for the presence of the miR-96 sequence in the genome of other plant species (NCBI database) did not gave positive matches.

3.1.2. Accumulation of miR-96 in *dcl* mutants.

Small RNA northern blot analysis indicated that miR-96 accumulates at a lower level in *dcl3* and *dcl4* mutants compared to wild-type plants, whereas its accumulation in *dcl1* plants was comparable to that in wild-type plants (Figure 10C). This finding supports a DCL3 and/or DCL4 dependency for production of miR-96, as it is the case of “young” *MIR* genes.

3.1.3. Production and molecular characterization of rice plants constitutively expressing miR-96.

The miR-96 precursor sequence was cloned into the plant expression vector pCAMBIA1300 (Figure 11A), and its functionality was verified by agroinfiltration in *N. benthamiana* leaves. The expected miR-96-5p small RNA sequences were detected in *N. benthamiana* leaves that have been agroinfiltrated with the miR-96 precursor (Figure 11B). Trangenic rice lines were generated and characterized for transgene insertion

(Figure 11C) and accumulation of miR-96-5p species and precursor transcripts (Figure 11D, E). Trangene expression was monitored in successive generations (up to the T3 generation). The transgene copy number was estimated by qPCR in the T3 generation. Finally, the transgenic plants overexpressing miR-96 were normal in growth and development (Figure 11F). Three independently generated transgenic lines harboring a single copy of the transgene (lines 2, 5 and 7; Table 6) were used for further analysis.

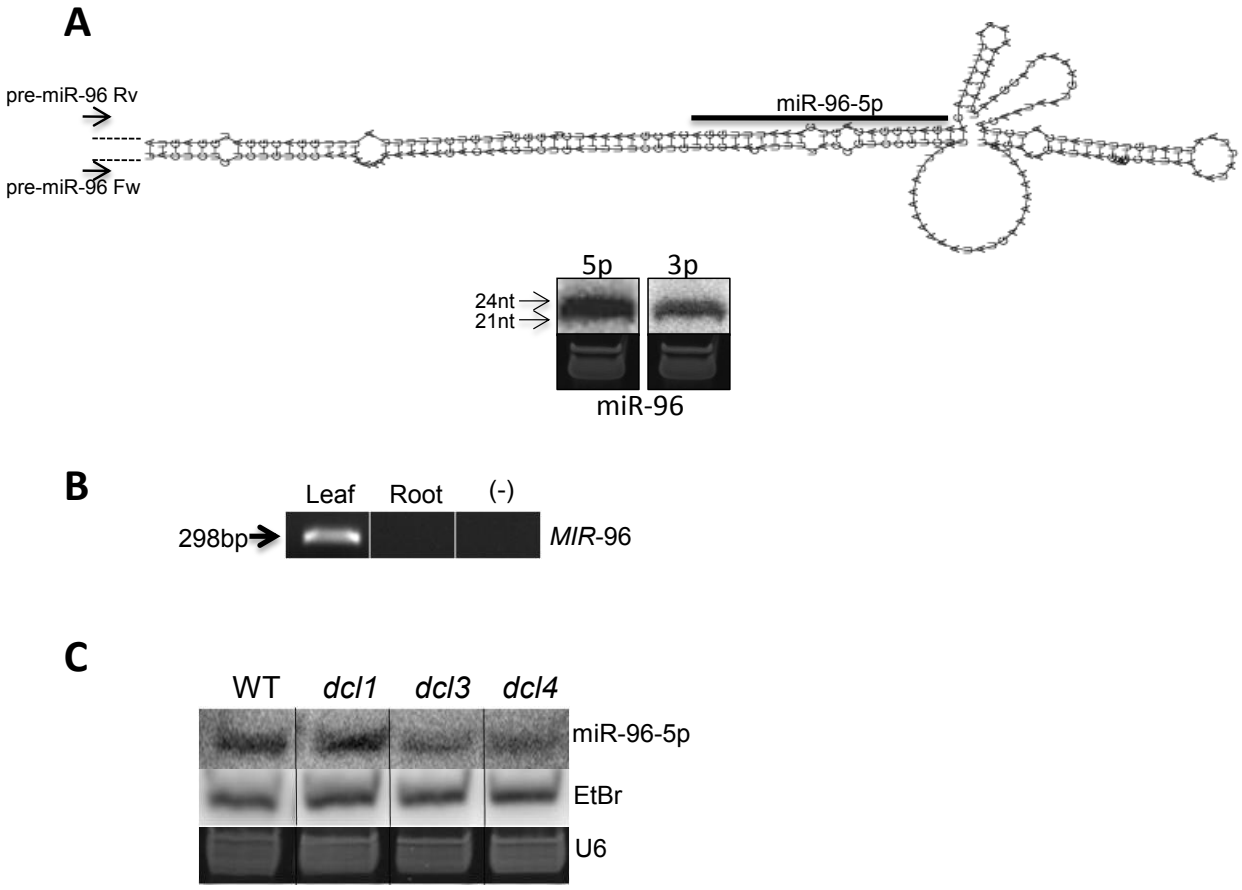


Figure 10. Experimental validation of miR-96. (**A)** Predicted hairpin structure of the miR-96 precursor structure. Small RNA sequences recovered from small RNA sequencing datasets (Campo et al., 2013) are represented by black bars. Arrows (Fw, Rv) indicate the primers used for amplification in B. Northern blot analysis of small RNAs obtained from rice leaves, and corresponding ethidium bromide staining, is shown in the lower panel. Here, 200 ug of total RNA were used to obtain the small RNA fraction, were hibridized with a synthetic oligonucleotides probes, complementary to the miRNA duplex osa-miR-96 sequences. (**B)** Detection of miR-96 precursor expression by reverse transcription polymerase chain reaction (RT-PCR). RNAs from leaves and roots of 21-old rice plants were analyzed. (-), RT-PCR reaction without reverse transcriptase. Sequencing of the PCR-amplified DNA fragment encompassing the miR-96 precursor sequence confirmed the specific amplification of this precursor. **(C)** Northern blot analysis of miR-96-5p in rice mutants affected in miRNA biogenesis, the *dcl1*, *dcl3* and *dcl4* mutants. The same blot was stripped and re-probed with the U6 probe. The lower panel shows the ethidium bromide staining of RNA samples.

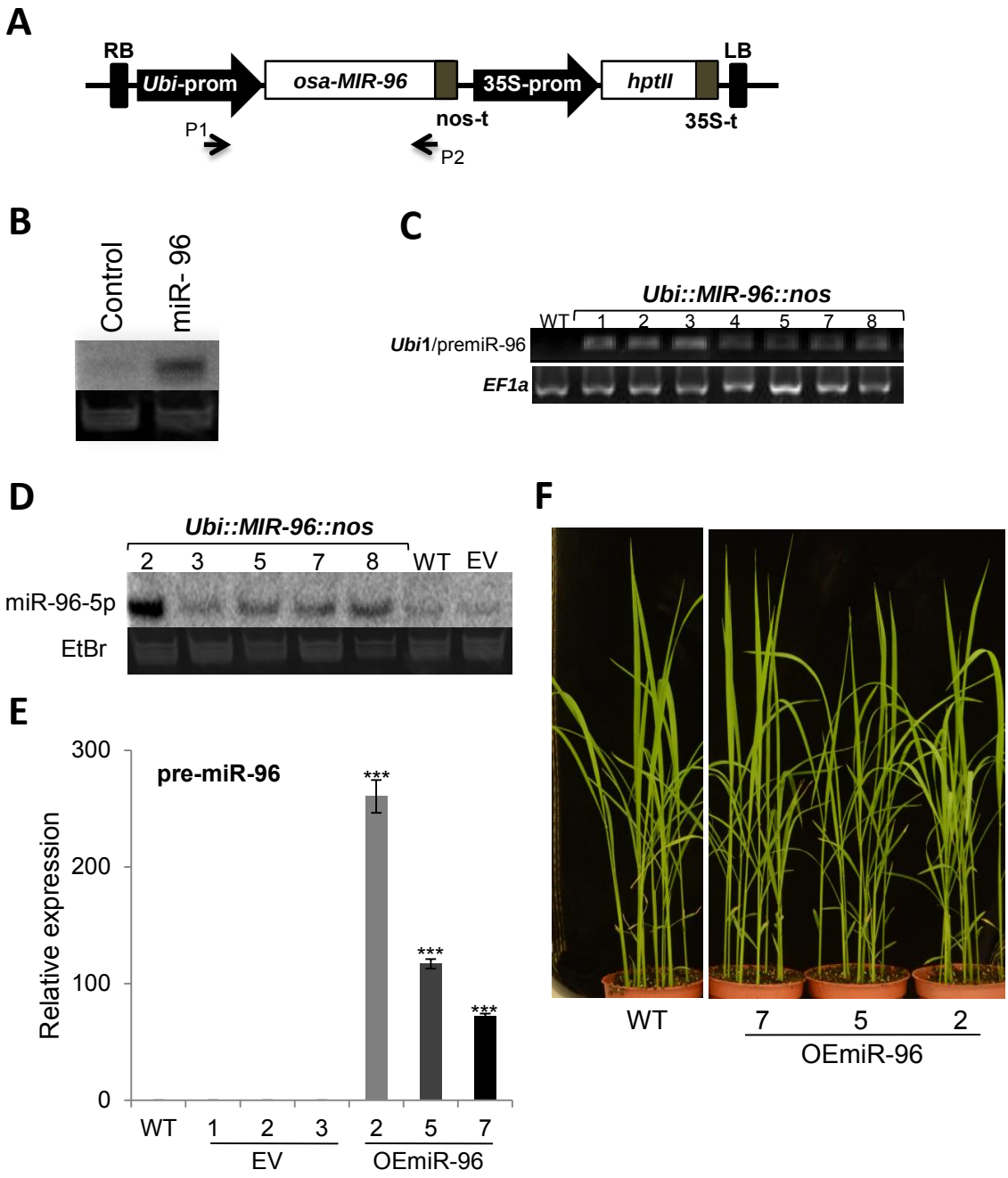


Figure 11. Molecular characterization of rice plants overexpressing miR-96. (A) Schematic diagram of the plant expression vector used for constitutive expression of miR-96 in rice. Expression of the miR-96 precursor is driven by the maize ubiquitin promoter (*Ubi-prom*) and the *nopaline synthase* terminator (*nos-t*). The *hptII* (*hygromycin phosphotransferase*) gene encoding resistance to hygromycin serves as a selectable marker for rice transformation. *35S-prom*, 35S promoter from the cauliflower mosaic virus. RB, right border; LB, left border. Arrows indicate the primers used for PCR amplification in C. **(B)** Functional analysis of the miR-96 precursor in *N. benthamiana* leaves (*rdr6IR* line). The miR-96-5p sequence produced from this precursor was detected by agroinfiltration of *N. benthamiana* leaves, followed by northern blot analysis using oligonucleotides complementary to miR-96-5p. No signal was detected in control leaves agroinfiltrated with the empty pCAMBIA vector (control). **(C)** Transgene integration and integrity was confirmed by PCR analysis of genomic DNA of independent transgenic lines. Primers used for PCR located at the 3' end of the *Ubiquitin* promoter (*Ubi1*) and miR-96 precursor (downstream of the sequence forming the stem-loop structure). As a negative control WT plants were used. **(D)** Accumulation of miR-96-5p species in leaves of independently generated transgenic rice lines overexpressing miR-96 plants. Results obtained for representative transgenic lines with different levels of miR-96 accumulation are

shown. **(E)** Accumulation of pre-miR-96 transcripts in leaves of plants determined by RT-PCR**.** WT (wild-type), EV (transgenic rice lines harbouring the empty vector). Statistical significance was determined by ANOVA (***, P ≤ 0.001). The histogram shows the mean ± SD. **(F)** Appearance of 3 week-old miR-96 and wild-type plants grown under controlled greenhouse conditions.

	Lines	Number of copies
OEmiR-96	2	1,04
	5	1,06
	7	0,97
EV	1	0,96
	2	0,99
	3	1,07

Table 6. Analysis of number of copies for the transgen in pre-miR-96 overexpressing plantas by qPCR using the *sucrose phosphate synthase* (*SPS*) gene like endogenous reference gene (Yang et al., 2004).

3.1.4. Constitutive expression of miR-96 increases susceptibility to infection by *M. oryzae*.

Three independen transgenic rice lines constitutively expressing miR-96 and control plants were spray-inoculated with a spore suspension of *M. oryzae* ($1x10^5$spores/ml). The OE miR-96 lines were found to be more susceptible to infection than control plants as determined by visual inspection of infected leaves, quantification of lesion area and fungal biomass (Figure 12A-C). Differences were, however, observed in susceptibility among the three lines here assayed. The transgenic lines 2 and 7 exhibited relatively higher blast disease symptoms compared to line 5. The degree of susceptibility was not correlated with the level of miR-96 accumulation (e.g. line 2 accumulated higher levels of miR-64; see Figure 11D).

Finally, we found that *PR1b* expression was induced at a lower level than control (empty vector) plants upon inoculation with *M. oryzae* (Figure 12D). The phenotype of susceptibility that is observed in miR-96 plants might be the consequence of a weaker defense response in this plants which might in turn decrease the plant's ability to resist pathogen infection. From these results, it is concluded that miR-96 overexpression negatively regulates disease resistance in rice plants.

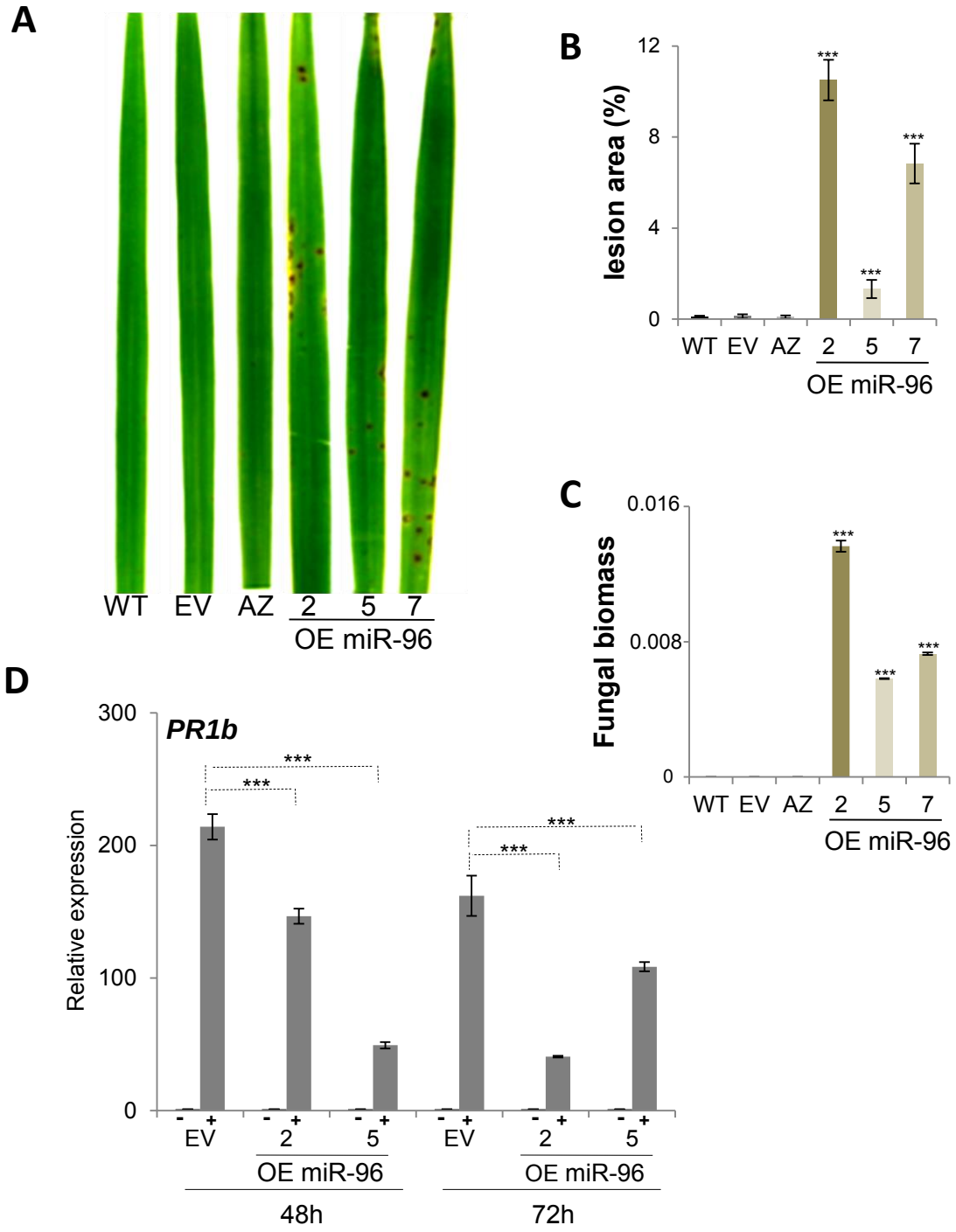


Figure 12. Susceptibility of rice plants overexpressing miR-96 to infection by the rice blast fungus *Magnaphorte oryzae*. (A) Susceptibility was tested in three independent transgenic miR-96 (homozygous, T3 generation), empty vector (EV, three independent homozygous lines), and wild-type (WT, *Nipponbare*; AZ, azygous controls segregated from the T0) plants. Plants at the three-leaf stage (15 plants/genotype) were inoculated with *M. oryzae* spores ($1x10^5$ spores/ml). At 7 days post-inoculation, disease symptoms at the second leaf were evaluated. Results are from one out of three independent infection experiments which gave similar results. **(B)** Percentage of the leaf area showing blast lesions (7dpi). **(C)**. Quantification of *M. oryzae* DNA by qPCR at 3 days post-inoculation using specific primers of the *M. oryzae 28S* gene. Values are fungal DNA levels were normalized against the rice *UBIQUITIN* gene. **(D)** Expression of defense marker gene *PR1b* in OEpremiR-96 plants and wild-type plants. Three-week old OE premiR-96plants were inoculated with *Magnaphorte oryzae spores*. Expression analyses were carried out by RT-qPCR 48 and 72 hours after inoculation. Mock-inoculated and inoculated plants are represented by black and grey bars,

respectively. As expected defense marker genes have a less or similar respond to infection in transgenic lines plants compared to control plants. Statistical significance was determined by ANOVA (***, P ≤0.001). Histograms show the mean ± SD.

3.1.5. Predicted targets of miR-96.

Five target genes were predicted for miR-96-5p and one gene was predicted as the target gene for miR-96-3p (Table 7). The psRNAtarget prediction software was used (http://plantgrn.noble.org/psRNATarget/). However, none of the putative target genes was supported by degradome analysis (Baldrich et al., 2015). Further studies are needed to confirm whether any of the predicted targets for this miRNA is a real target.

Predicted target genes for miR-96						
miRNA	**Predicted target**	**Locus ID**	**Score**	**Alignment**	**Cleavage position**	**Degradome analysis**
miR-96 (5p)	DNA polymerase I	Os03g12610	2.0	||||| |||||||||||	2106-2127	NO
miR-96 (5p)	Protein synthesis inhibitor I	Os01g06740	3.0	||||| o||||||||||||	1200-1222	NO
miR-96 (5p)	Xylanase inhibitor	Os01g71060	3.0	||||| o||||||||||||	1248-1270	NO
miR-96 (5p)	Expressed protein	Os03g24730	3.0	||||| o||||||||||||	90-112	NO
miR-96 (5p)	Chloroplast outer envelope protein 86	Os12g09570	3.0	|||| o||||||||||||	2703-2725	NO
miR-96 (3p)	Protein disulfide isomerase (PDI)-like protein	Os03g29190	2.5	|||| |||||| ||||||||	159-183	NO

Table 7. Predicted target genes for miR-96. Target prediction was done using the psRNAtarget program. The circles indicate G::U pairing.

3.2. Studies on miR98

In an effort to identify new miRNAs from rice potentially involved in resistance to blast in rice plants, other miRNA candidates were investigated in this work. At present, the characterization of these candidates is in a less advanced stage. They are: miR-98 and miR-203.

Following the same experimental approaches above indicated, the accumulation of the two small RNAs mapping opposite to each other in the precursor structure has been demonstrated (in this case, the small RNA fraction obtained from 200 µg of total RNA was analyzed) (Figure 13A). Precursor transcripts containing miR-98-5p and miR-98-3p accumulated in rice leaves (Figure 13B). Studies to determine *dcl* dependency for production of these small RNAs have not been carried out yet. Transgenic rice lines constitutively expressing miR-98 were generated and characterized for transgene integration and expression (Figure 14C-E). Transgenic lines containing a single copy of the transgene were identified (Table 8). When grown under controlled greenhouse conditions, the OE miR-98 plants showed no alteration in growth compared to wild type plants (Figure 14F).

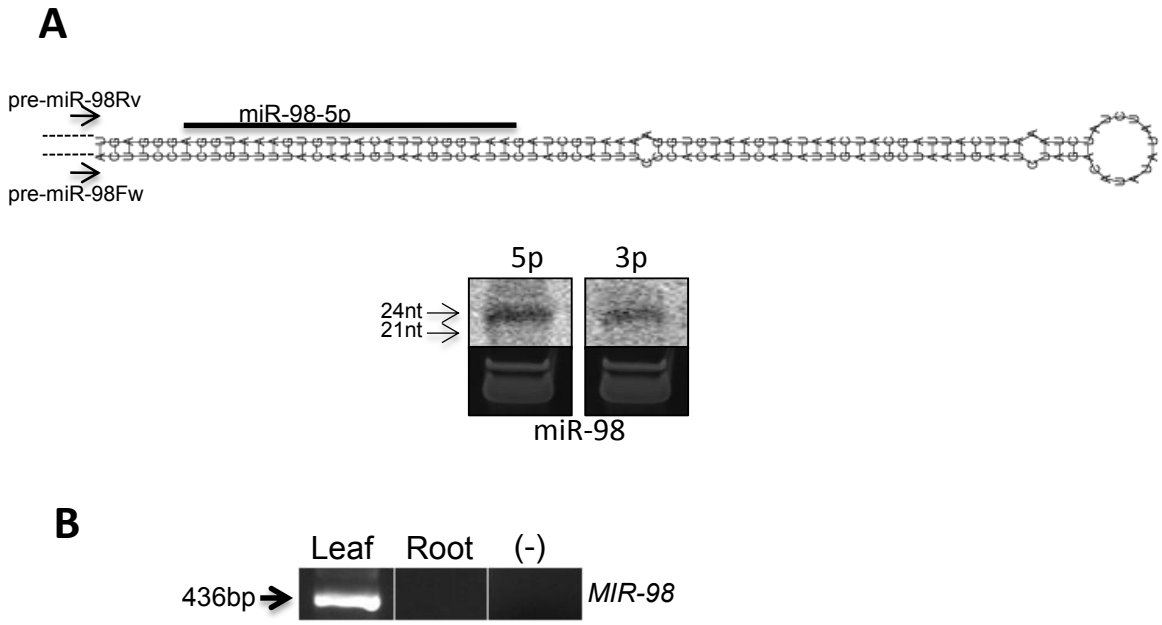


Figure 13. Experimental validation of miR-98. (**A)** Predicted hairpin structure of the miR-98 precursor structure. Small RNA sequences recovered from small RNA sequencing datasets (Campo et al., 2013) are represented by black bars. Arrows (Fw, Rv) indicate the primers used for amplification in B. Northern blot analysis of small RNAs obtained from rice leaves, and corresponding ethidium bromide staining, is shown in the lower panel. Here, 200 ug of total RNA were used to obtain the small RNA fraction, were hybridized with a synthetic oligonucleotides probes, complementary to the miRNA duplex osa-miR-98 sequences. (**B)** Detection of miR-98 precursor expression by reverse transcription polymerase chain reaction (RT-PCR). RNAs from leaves and roots of 21-old rice plants were analyzed. (-), RT-PCR reaction without reverse transcriptase. Sequencing of the PCR-amplified DNA fragment encompassing the miR-98 precursor sequence confirmed the specific amplification of this precursor.

	Lines	Number of copies
OEmiR-98	1	1,03
	2	1,05
	3	0,96
EV	1	0,96
	2	0,99
	3	1,07

Table 8. Determination of transgene copy number in plants overexpressing miR-98 by qPCR using the sucrose phosphate synthase (SPS) gene as the endogenous reference gene (Yang et al., 2004).

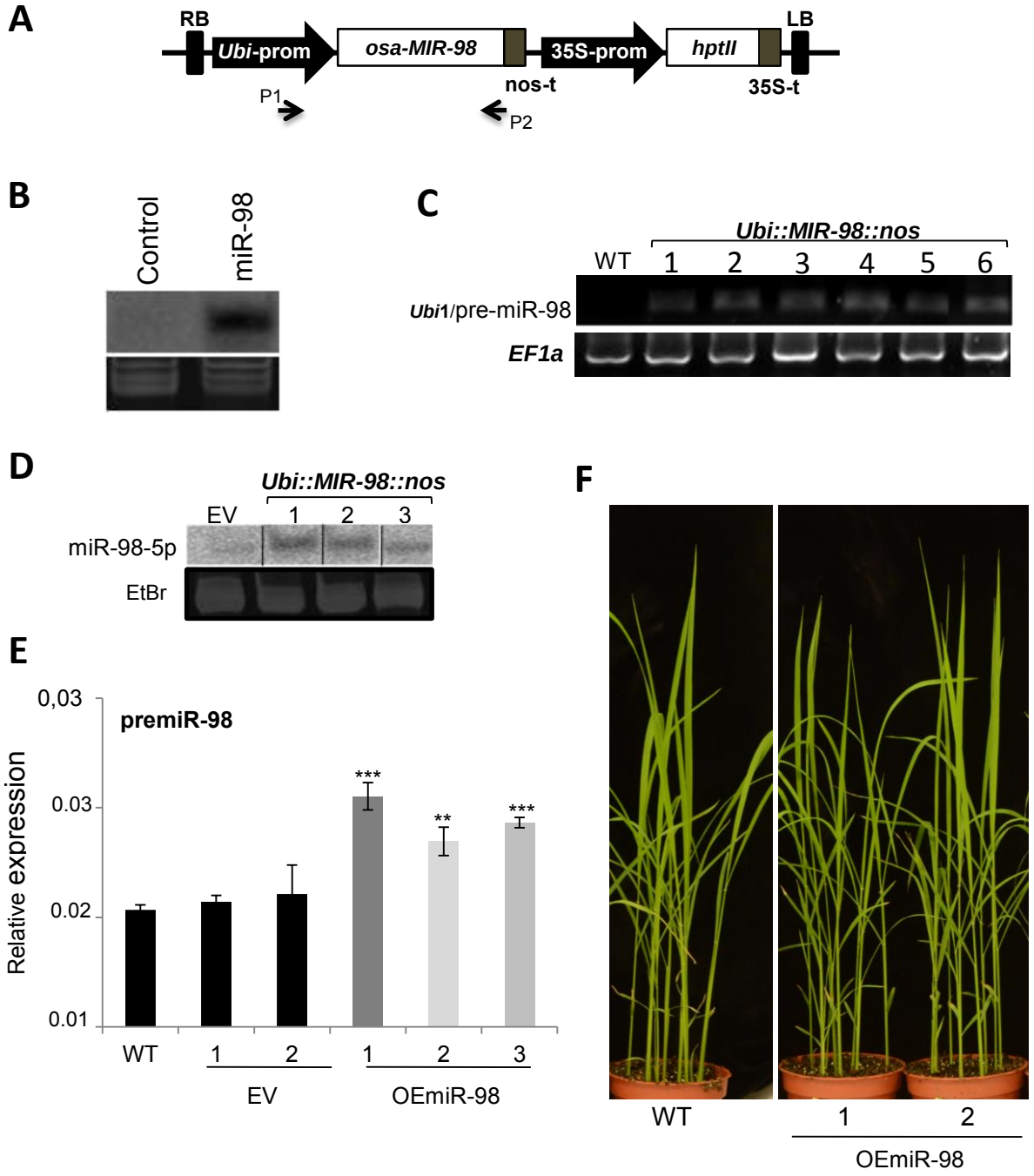


Figure 14. Molecular characterization of rice plants overexpressing miR-98. (A) Schematic diagram of the plant expression vector used for constitutive expression of miR-98 in rice. Expression of the miR-98 precursor is driven by the maize ubiquitin promoter (*Ubi-prom*) and the *nopaline synthase* terminator (*nos-t*). The *hptII* (*hygromycin phosphotransferase*) gene encoding resistance to hygromycin serves as a selectable marker for rice transformation. *35S-prom*, 35S promoter from the cauliflower mosaic virus. RB, right border; LB, left border. Arrows indicate the primers used for PCR amplification in C. **(B)** Functional analysis of the miR-98 precursor in *N. benthamiana* leaves (*rdr6IR* line). The miR-98-5p sequence produced from this precursor was detected by agroinfiltration of *N. benthamiana* leaves, followed by northern blot analysis using oligonucleotides complementary to miR-98-5p. No signal was detected in control leaves agroinfiltrated with the empty pCAMBIA vector (control). **(C)** Transgene integration and integrity was confirmed by PCR analysis of genomic DNA of independent transgenic lines. Primers used for PCR located at the 3' end of the *Ubiquitin* promoter (*Ubi1*) and miR-98 precursor (downstream of the sequence forming the stem-loop structure). As controls WT plants were used. **(D)** Accumulation of miR-98-5p species in leaves of independently generated transgenic rice lines overexpressing miR-98 plants. Results obtained for representative transgenic lines with different levels of miR-98 accumulation are

shown. **(E)** Accumulation of pre-miR-98 transcripts in leaves of plants determined by RT-PCR**.** WT, wild-type, EV, transgenic rice lines harbouring the empty vector. Statistical significance was determined by ANOVA (**, P ≤ 0.01; ***, P ≤ 0.001). The histogram shows the mean ± SD. **(F)** Appearance of 3 week-old miR-98 and wild-type plants grown under controlled greenhouse conditions.

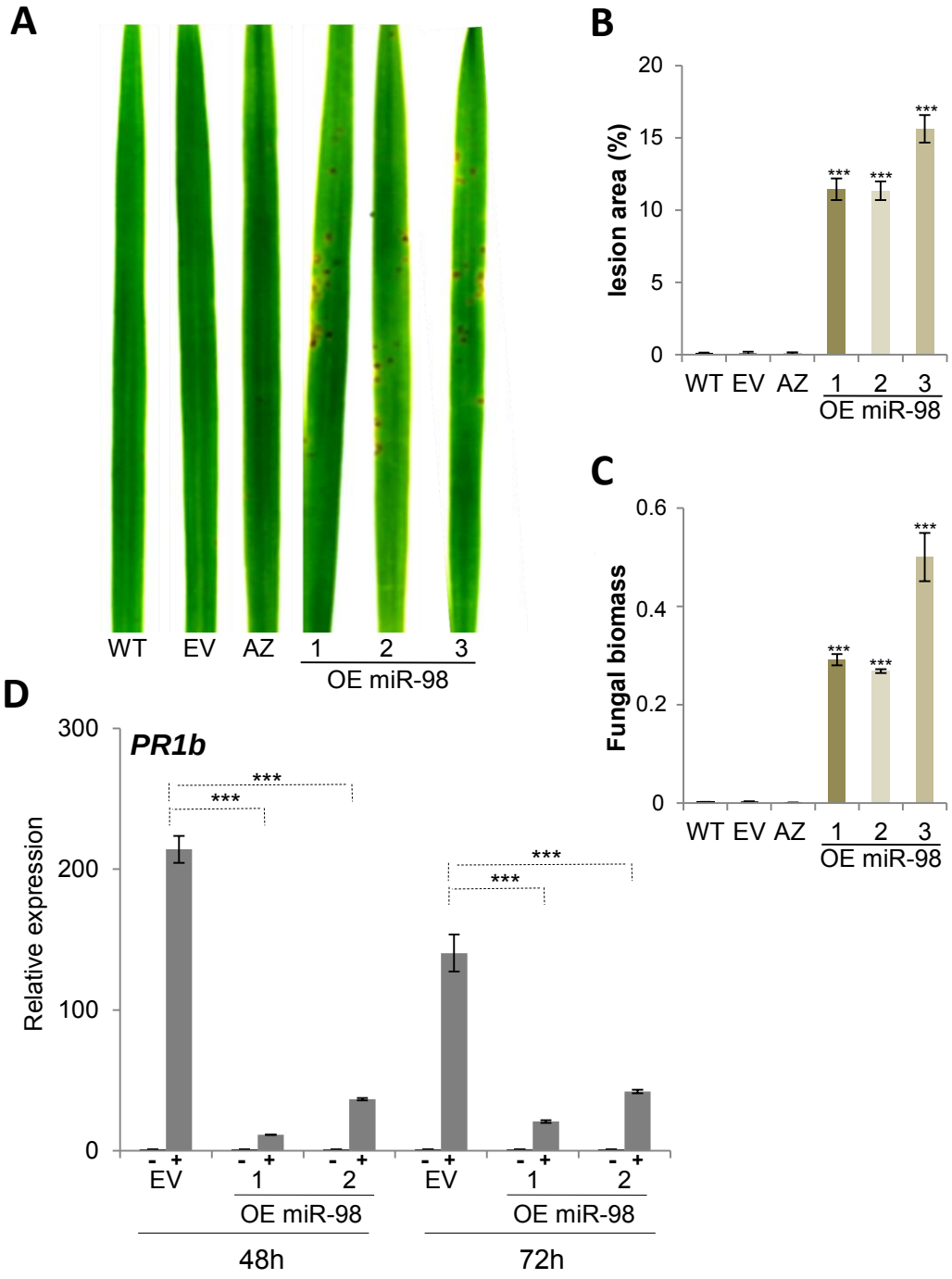


Figure 15. Susceptibility of rice plants overexpressing miR-98 to infection by the rice blast fungus *Magnaphorte oryzae*. (A) Susceptibility was tested in three independent transgenic miR-98 (homozygous, T3 generation), empty vector (EV, three independent homozygous lines), and wild-type (WT, *Nipponbare*; AZ, azygous controls segregated from the T0) plants. Plants at the three-leaf stage (15 plants/genotype) were inoculated with *M. oryzae* spores ($1x10^5$ spores/ml). At 7 days post-inoculation, disease symptoms at the second leaf were evaluated. Results are from one out of three independent infection experiments which gave similar results. **(B)** Percentage of the leaf area showing blast lesions (7dpi). **(C)** Quantification of *M.*

oryzae DNA by qPCR at 3 days post-inoculation using specific primers of the *M. oryzae 28S* gene. Values are fungal DNA levels were normalized against the rice *UBIQUITIN* gene. **(D)** Expression of defense marker gene *PR1b* in OEpremiR-98 plants and wild-type plants. Three-week old OE premiR-98plants were inoculated with *Magnaphorte oryzae spores*. Expression analyses were carried out by RT-qPCR 48 and 72 hours after inoculation. Mock-inoculated and inoculated plants are represented by black and grey bars, respectively. As expected defense marker genes have a less or similar respond to infection in transgenic lines plants compared to control plants. Statistical significance was determined by ANOVA (***, P ≤0.001). Histograms show the mean ± SD.

Constitutive expression of miR-98 increased susceptibility to infection by *M. oryzae* compared to control plants (wild-type, transgenic lines harbouring the empty vector, and azygous plants), which was further assessed by quantifying the leaf area with lesions and the amount of fungal DNA in the infected leaves (Figure 15 A-C). Consistent with the phenotype of susceptibility, a lower level of *PR1b* induction was observed in OE miR-98 plants compared to control plants (Figure 15D).

An important number of genes were predicted as miRNA targets of miR-98, both miR-98-5p and miR-98-3p (Table 9). The predicted genes encode proteins associated with various biological processes, some of them having a function coherent with the plant response to pathogen infection (e.g. triacylglycerol lipase, glutarredoxins, MLO-like protein). In Arabidopsis, the *PAD4* gene encoding a triacylglycerol lipase, is required for expression of multiple defense responses including camalexin production and *PR1* gene expression during infection with the bacterial pathogen *P. syringae* (Jirage et al., 1999). To provide experimental evidence of miR-75 target genes we searched in the degradome sequencing datasets. Eight predicted target transcripts had degradome tags, this finding reinforcing the idea that miR-98 is a real miRNA (Table 9). However, additional experimental validation is needed to determine how many of these targets are genuinely regulated by miR-98 during pathogen infection.

Predicted target genes for miR-98						
miRNA	Predicted target	Locus ID	Score	Alignment	Cleavage position	Degradome analysis
miR-98 (5p)	Triacylglycerol lipase	Os03g22670	1.5	||||o|||||||||oo||	1083	Validated
miR-98 (5p)	Vesicle-associated membrane protein	Os12g44250	1.0	||||o|||||||||o|||	861	Validated
miR-98 (5p)	Cytochrome P450 78A11	Os03g40600	3.0	||||o|||| |||||||ooo|	708	Validated
miR-98 (5p)	D-mannose binding lectin family protein	Os09g28180	2.0	||||o|||| |||||||o|||	3824	Validated
miR-98 (5p)	Glutaredoxin 2	Os02g52900	2.5	||| | |||||o|||||||||	1981	Validated
miR-98 (5p)	WRKY34 transcription factor	Os02g43560	1.5	|||||o||||||o|||||o|||	2745	Validated
miR-98 (5p)	Ribosome inactivating protein	Os10g42060	1.0	|||||o|||||||||||o|||	917	Validated
miR-98 (5p)	Expressed protein	Os01g68230	1.5	|||||o||||||||o|||o|||	2339	Validated
miR-98 (5p)	AIR12	Os03g09880	1.5	||||||||||||||o||||	781-804	NO
miR-98 (5p)	Expressed protein	Os04g32610	1.5	|||o|||||||||||o||||	236-259	NO
miR-98 (5p)	GPI-anchored protein	Os06g19990	1.5	|||o|||||||||||o||||	659-682	NO
miR-98 (5p)	Expressed protein	Os05g41190	1.5	||o||||||||||||o||||	959-982	NO
miR-98 (5p)	Aspartic proteinase nepenthesin-2	Os04g58070	1.5	|||o|||||||||||o||||	1502-1525	NO
miR-98 (5p)	Ankyrin-like protein	Os02g29140	1.5	|||o|||||||||||o||||	1527-1550	NO
miR-98 (5p)	Indole-3-acetate beta-glucosyltransferase	Os09g34250	1.5	|||o|||||||||||o||||	1758-1781	NO
miR-98 (5p)	RAB, member of RAS oncogene family-like 3	Os10g04580	1.5	|||o|||||||||||o||||	1810-1833	NO
miR-98 (5p)	Phosphatidylcholine-sterol acyltransferase	Os03g52010	1.5	|||o|||||||||||o||||	1773-1796	NO
miR-98 (5p)	Expressed protein	Os08g04920	1.5	|||o|||||||||||o||||	1912-1935	NO
miR-98 (5p)	OsGrx_S15.1 - glutaredoxin subgroup II	Os01g34620	1.5	|||o|||||||||||o||||	473-496	NO
miR-98 (5p)	Dynamin-related protein 1C	Os09g39960	1.5	|||o|||||||||||o||||	2324-2347	NO
miR-98 (5p)	Glycosyl transferase	Os08g04300	1.5	|||o|||||||||||o||||	2074-2097	NO
miR-98 (5p)	Retrotransposon protein	Os09g09820	1.5	|||o|||||||||||o||||	753-776	NO
miR-98 (5p)	Expressed protein	Os12g18729	1.5	|||o|||||||||||o||||	3162-3185	NO
miR-98 (5p)	Expressed protein	Os08g19114	1.5	|||o|||||||||||o||||	2023-2046	NO
miR-98 (5p)	Zinc finger, C3HC4 type family protein	Os06g23274	1.5	|||o|||||||||||o||||	4567-4590	NO
miR-98 (5p)	Steroid dehydrogenase let-767	Os04g02620	1.5	||oo||||||||||o||||	1617-1640	NO
miR-98 (5p)	Ribose-phosphate pyrophosphokinase 4	Os02g48390	1.5	||oo||||||||||o||||	1684-1707	NO
miR-98 (5p)	MLO-like protein 4	Os11g07912	1.5	|o|o||||||||||o||||	2147-2170	NO
miR-98 (5p)	Soluble starch synthase 1, chloroplast	Os06g06560	1.5	|o|o||||||||||o||||	2653-2676	NO
miR-98 (5p)	B3 DNA binding domain containing protein	Os01g13300	1.5	||o||||||||||o||||	2150-2173	NO
miR-98 (5p)	Chlorophyll a-b binding protein, chloroplast	Os03g39610	2.5	|||o||||||||||o|||o	1429-1452	NO
miR-98 (5p)	Dihydroflavonol-4-reductase	Os07g41060	2.5	|||o|||||o|||||||o	558-581	NO
miR-98 (5p)	Farnesyltransferase/geranylgeranyltransferase type I alphasubunit	Os09g33930	2.5	|||o||||||||||o|||o	1390-1413	NO
miR-98 (5p)	Cytochrome P450 89A2	Os08g05620	2.5	|||o|||o|||||||o||||	1675-1698	NO
miR-98 (5p)	Expressed protein	Os01g68230	2.5	|||o|||o|||||||o||||	1735-1758	NO
miR-98 (5p)	Gamma-glutamyltranspeptidase 1	Os04g38450	2.5	|||o|||o|||||||o||||	2110-2133	NO
miR-98 (5p)	Ureide permease 4	Os12g31860	2.5	|||o||||||||||o||||	1876-1899	NO
miR-98 (5p)	Ubiquitin-conjugating enzyme E2-17 kDa	Os09g12230	2.5	||oo||||||||||o|||o	994-1017	NO
miR-98 (3p)	Hypothetical protein	Os09g02620	0.0	|||||||||||||||||||	158-181	NO
miR-98 (3p)	Ubiquitin-conjugating enzyme E2-17 kDa	Os09g12230	0.0	|||||||||||||||||||	1120-1143	NO
miR-98 (3p)	F-box domain containing protein	Os02g44990	0.0	|||||||||||||||||||	1895-1918	NO
miR-98 (3p)	Expressed protein	Os08g08160	0.0	o||||||||||||||||||	1581-1604	NO
miR-98 (3p)	GPI-anchored protein	Os06g19990	0.0	|||||||||||||||||||	728-751	NO
miR-98 (3p)	Early nodulin 75 protein	Os05g13940	0.0	|||||||||||||||||||	1805-1828	NO
miR-98 (3p)	Protein app1	Os03g56930	0.0	|||||||||||||||||||	1045-1068	NO
miR-98 (3p)	3-ketoacyl-CoA synthase	Os04g02640	1.0	||||||||||||||||||o	1723-1746	NO
miR-98 (3p)	Expressed protein	Os01g15520	1.5	||||||||||||||||o|||	960-983	NO
miR-98 (3p)	Expressed protein	Os05g50440	1.5	||||||||||||||||o|||	911-934	NO
miR-98 (3p)	Dihydroflavonol-4-reductase	Os07g41060	1.5	||||||||||||||||o|||	653-676	NO
miR-98 (3p)	Protein farnesyltransferase/geranylgeranyltransferase type I alphasubunit	Os09g33930	1.5	||||||||||||||||o|||	1503-1526	NO
miR-98 (3p)	CAAX prenyl protease 1	Os02g45650	1.5	||||||||o||||||||	1783-1806	NO
miR-98 (3p)	Terpene synthase 7	Os07g11790	1.5	||||||||||||||||o|||	1722-1745	NO
miR-98 (3p)	Phosphatidylcholine-sterol acyltransferase precursor	Os03g52010	1.5	||||||||||||||||o|||	1884-1907	NO
miR-98 (3p)	Beclin-1-like protein	Os03g44200	1.5	||||||||||||||||o|||	2054-2077	NO
miR-98 (3p)	Gamma-glutamyltranspeptidase 1	Os04g38450	1.5	||||||||||||||||o|||	2223-2246	NO
miR-98 (3p)	Expressed protein	Os06g43980	1.5	||||||||||||||||o|||	1644-1667	NO
miR-98 (3p)	Expressed protein	Os12g13174	1.5	||||||||||||||||o|||	1038-1061	NO
miR-98 (3p)	Flavonol synthase/flavanone 3-hydroxylase	Os06g07914	1.5	o||||||||||||||||o|||	1445-1468	NO
miR-98 (3p)	Cytochrome P450 89A2	Os08g05620	1.5	o||||||||||||||||o|||	1788-1811	NO
miR-98 (3p)	Expressed protein	Os08g31910	1.5	o||||||||||||||||o|||	407-430	NO
miR-98 (3p)	Auxin-independent growth promoter	Os06g17390	1.5	o||||||||||||||||o|||	2710-2733	NO
miR-98 (3p)	OsWRKY34 transcription factor	Os02g43560	1.5	o||||||||||||||||o|||	2844-2867	NO
miR-98 (3p)	RING-H2 finger protein ATL5I	Os06g07100	1.5	||||||||||||||||o|||	1637-1660	NO
miR-98 (3p)	Steroid dehydrogenase let-767	Os04g02620	1.5	||||||||||||||||o|||	1732-1755	NO
miR-98 (3p)	Glucosyltransferase-like protein	Os04g20400	1.5	||||||||||||||||o|||	1522-1545	NO
miR-98 (3p)	Expressed protein	Os05g51140	1.5	||||||||||||||||o|||	1635-1658	NO
miR-98 (3p)	Retrotransposon protein	Os09g09820	1.5	||||||||||||||||o|||	866-889	NO
miR-98 (3p)	Expressed protein	Os07g01130	1.5	||||||||||||||||o|||	4239-4262	NO
miR-98 (3p)	Zinc finger, C3HC4 type family protein	Os06g23274	1.5	||||||||||||||||o|||	4719-4742	NO
miR-98 (3p)	Glycosyl transferase	Os12g37510	1.5	o||||||||||||||||o|||	1630-1653	NO
miR-98 (3p)	Hypothetical protein	Os05g42360	1.5	||||||||||||o|||	66-89	NO
miR-98 (3p)	Ureide permease 4	Os12g31860	2.5	||||||||||||||o||o	1977-2000	NO
miR-98 (3p)	Hypothetical protein	Os09g14560	2.5	|||||||||||||||o|||	119-142	NO
miR-98 (3p)	Glycoprotein 3-alpha-L-fucosyltransferase A	Os08g36840	2.5	|||||||||||||||o|||	1862-1885	NO
miR-98 (3p)	RING-H2 finger protein ATL2L	Os04g49160	2.5	o||||||o|||||||o|||	1140-1163	NO
miR-98 (3p)	Hypothetical protein	Os03g38689	2.5	o|||||||||||||||o|||	95-118	NO
miR-98 (3p)	Chlorophyll a-b binding protein, chloroplast	Os03g39610	2.5	||||||||||||||||o|||	1542-1565	NO
miR-98 (3p)	Aspartic proteinase nepenthesin-2	Os04g58070	2.5	||||||||||||||||o|||	1615-1638	NO
miR-98 (3p)	Expressed protein	Os08g19114	2.5	||||||||||||||||o|||	2119-2142	NO
miR-98 (3p)	Expressed protein	Os05g03574	2.5	||||||||||||||||o|||	729-752	NO
miR-98 (3p)	Expressed protein	Os05g43650	2.5	|||||||||||||||o||o	1603-1626	NO

Table 9. Predicted target genes for miR-98 and validation by degradome analysis. The targets validated by degradome were found into Baldrich et al. (2015) dataset. Target prediction was carried out using the psRNAtarget program. The circles indicate G::U pairing.

3.3. Studies on miR-203

The two small RNA species mapping to the miR-203 precursor structure were detected in rice leaves. Of them, miR-203-3p was more abundant than miR-2013-5p (Figure 16A, B). Precursor transcripts encompassing the two small RNAs mapping to the miR-203 precursor were also detected in rice leaves (Figure 16B).

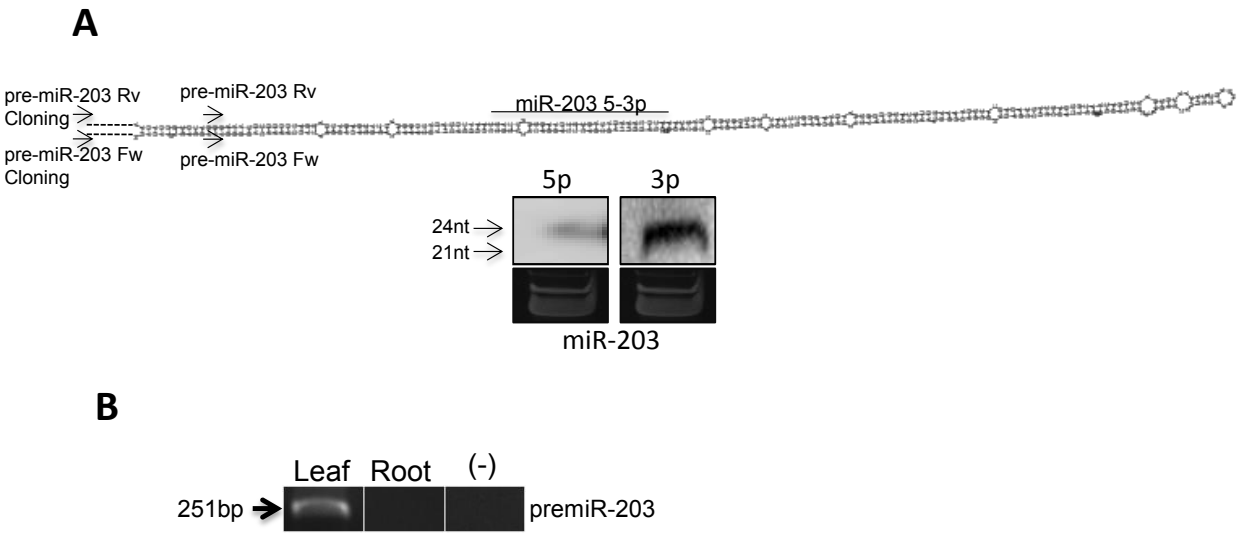


Figure 16. Experimental validation of miR-203. (A) Predicted hairpin structure of the miR-203 precursor structure. Small RNA sequences recovered from small RNA sequencing datasets (Campo et al., 2013) are represented by black bars. Arrows (Fw, Rv) indicate the primers used for amplification in B. Northern blot analysis of small RNAs obtained from rice leaves, and corresponding ethidium bromide staining, is shown in the lower panel. Here, 200 ug of total RNA were used to obtain the small RNA fraction, were hibridized with a synthetic oligonucleotides probes, complementary to the miRNA duplex osa-miR-203 sequences. (**B)** Detection of miR-203 precursor expression by reverse transcription polymerase chain reaction (RT-PCR). RNAs from leaves and roots of 21-old rice plants were analyzed. (-), RT-PCR reaction without reverse transcriptase. Sequencing of the PCR-amplified DNA fragment encompassing the miR-203 precursor sequence confirmed the specific amplification of this precursor.

The functionality of the cloned sequence containing the miR-203 precursor was assessed by agroinfiltration of *N. benthamiana* leaves (Figure 17A, B). Transgenic rice lines overexpressing miR-203 were produced and characterized for transgene integration (Figure 17C), expression (Figure 17D, E), and copy number (Table 10). No phenotypical differences were observed between OE miR-203 plants and wild-type plants (Figure 17F). When searching for the presence of miR-203 sequences in the genome of other plant species, this sequence was identified in the genome of *Elaeis guineensis* (palm oil) (see Table 1).

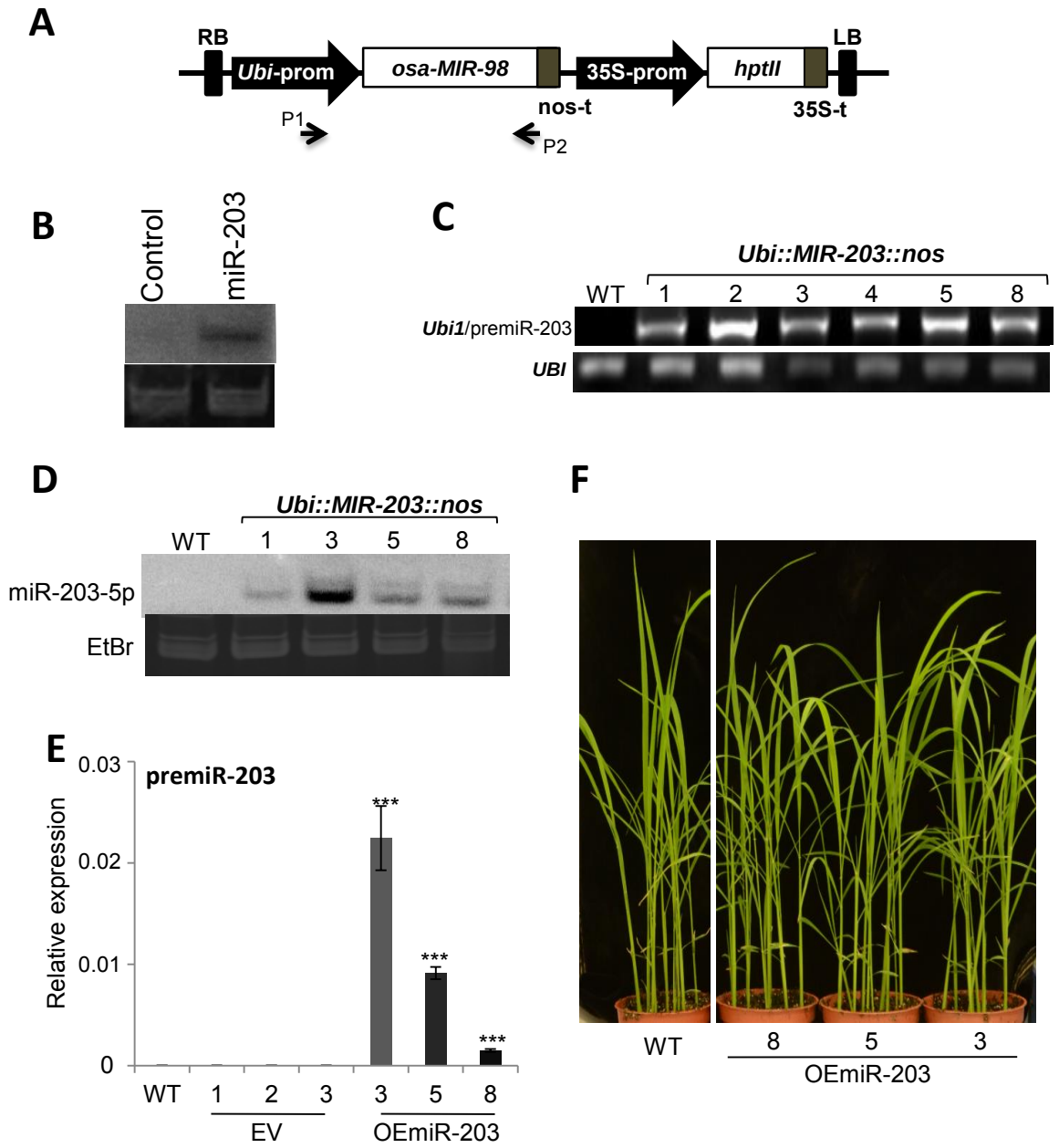


Figure 17. Molecular characterization of rice plants overexpressing miR-203. (A) Schematic diagram of the plant expression vector used for constitutive expression of miR-203 in rice. Expression of the miR-203 precursor is driven by the maize ubiquitin promoter (*Ubi-prom*) and the *nopaline synthase* terminator (*nos-t*). The *hptII* (*hygromycin phosphotransferase*) gene encoding resistance to hygromycin serves as a selectable marker for rice transformation. *35S-prom*, 35S promoter from the cauliflower mosaic virus. RB, right border; LB, left border. Arrows indicate the primers used for PCR amplification in C. **(B)** Functional analysis of the miR-203 precursor in *N. benthamiana* leaves (*rdr6IR* line). The miR-203-5p sequence produced from this precursor was detected by agroinfiltration of *N. benthamiana* leaves, followed by northern blot analysis using oligonucleotides complementary to miR-203-5p. No signal was detected in control leaves agroinfiltrated with the empty pCAMBIA vector (control). **(C)** Transgene integration and integrity was confirmed by PCR analysis of genomic DNA of independent transgenic lines. Primers used for PCR located at the 3' end of the *Ubiquitin* promoter (*Ubi1*) and miR-203 precursor (downstream of the sequence forming the stem-loop structure). As a control WT plants were used **(D)** Accumulation of miR-203-5p species in leaves of independently generated transgenic rice lines overexpressing miR-203 plants. Results obtained for representative transgenic lines with different levels of miR-203 accumulation are shown. **(E)** Accumulation of pre-miR-203 transcripts in leaves of plants determined by RT-PCR**.** WT, wild-type, EV, transgenic rice lines harbouring the empty vector. Statistical significance was determined by

ANOVA (***, P ≤ 0.001). The histogram shows the mean ± SD. **(F)** Appearance of 3 week-old miR-203 and wild-type plants grown under controlled greenhouse conditions

	Lines	Number of copies
OEmiR-203	3	1,10
	5	1,05
	8	1,04
EV	1	0,96
	2	0,99
	3	1,07

Table 10. Detection of transgene copy number in rice lines overexpressing miR-203 by qPCR using the *sucrose phosphate synthase* (*SPS*) gene like the endogenous reference gene (Yang et al., 2004).

OE miR-203 lines exhibited increased susceptibility to *M. oryzae* infection, lines 3 and 5 being the most susceptible ones (Figure 18 A-C). Furthermore, the OE-miR-203 plants had a reduced induction of *PR1b* expression upon pathogen challenge, which correlates with the observed phenotype of susceptibility in these plants (Figure 18D).

Finally, 2 target genes were predicted for each miR-2013-5p and miR-203-3p sequences using psRNAtarget server, but none of them could be validated by degradome analysis (Table 11). Further studies are then needed to validate the predicted target genes for miR-203.

Predicted target genes for miR-203						
miRNA	**Predicted target**	**Locus ID**	**Score**	**Alignment**	**Cleavage position**	**Degradome analysis**
miR-203 (5p)	Gibberellin 2-beta-dioxygenase 7	Os04g44150	2.0	|||||o||||||o||||	943-966	NO
miR-203 (5p)	Expressed protein	Os05g03972	3.0	||||o|||||o||||||oo	1908-1930	NO
miR-203 (3p)	HST	Os01g26160	2.5	|||||o|||||||oo||	3928-3951	NO
miR-203 (3p)	Glycosyltransferase	Os06g47340	3.0	o||o|o|||||||||||	1885-1908	NO

Table 11. Predicted targets for miR-203. Dataset of targets validated by degradome analysis were taken from Baldrich et al. (2015). Target prediction was perfomed with the psRNAtarget program. Circles indicate G::U pairing.

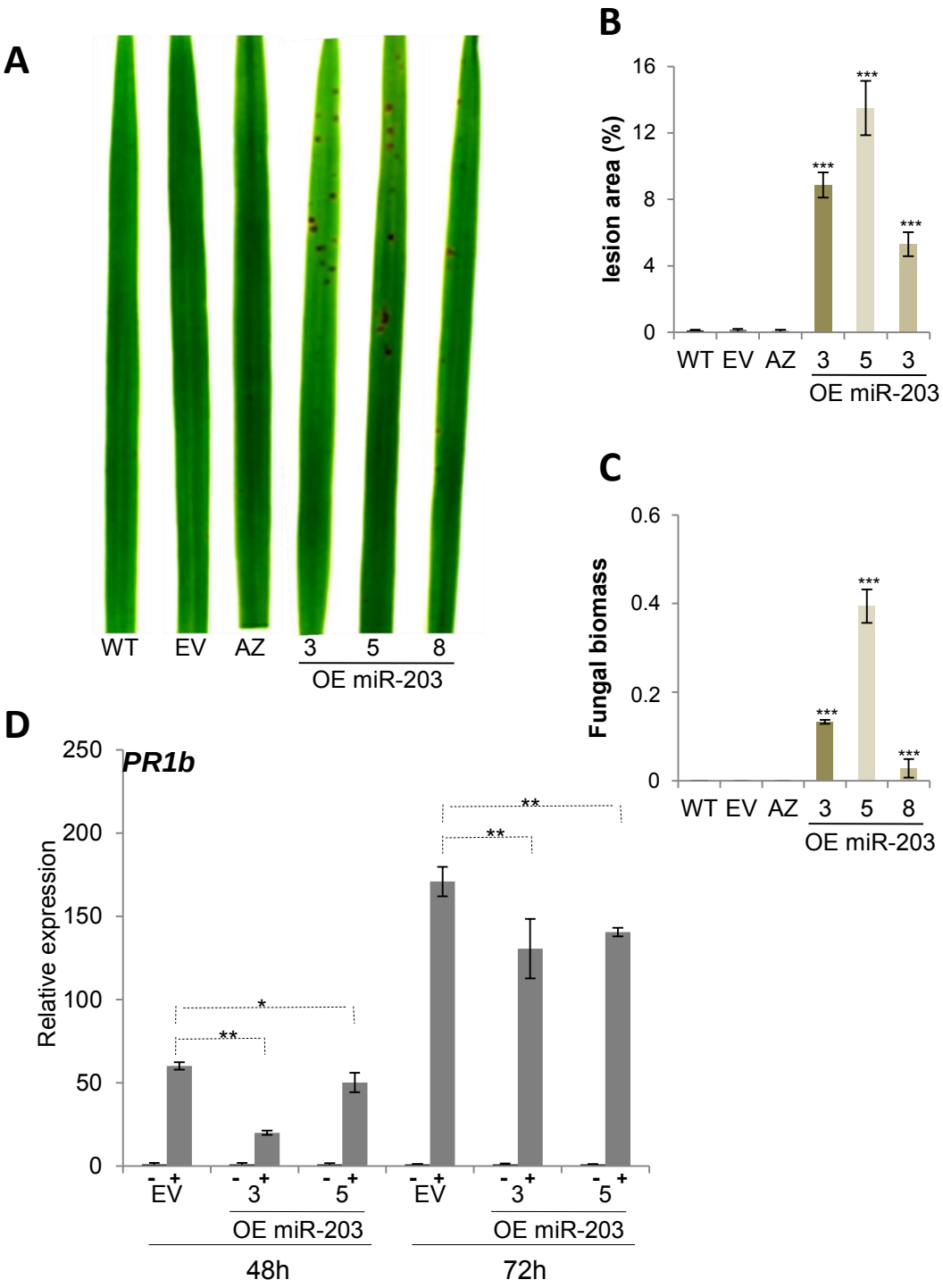


Figure 18. Susceptibility of rice plants overexpressing miR-203 to infection by the rice blast fungus *Magnaphorte oryzae*. **(A)** Susceptibility was tested in three independent transgenic miR-203 (homozygous, T3 generation), empty vector (EV, three independent homozygous lines), and wild-type (WT, *Nipponbare*; AZ, azygous controls segregated from the T0) plants. Plants at the three-leaf stage (15 plants/genotype) were inoculated with *M. oryzae* spores ($1x10^5$ spores/ml). At 7 days post-inoculation, disease symptoms at the second leaf were evaluated. Results are from one out of three independent infection experiments which gave similar results. **(B)** Percentage of the leaf area showing blast lesions (7dpi). **(C)** Quantification of *M. oryzae* DNA by qPCR at 3 days post-inoculation using specific primers of the *M. oryzae 28S* gene. Values are fungal DNA levels were normalized against the rice *UBIQUITIN* gene. **(D)** Expression of defense marker gene *PR1b* in OEpremiR-203 plants and wild-type plants. Three-week old OE premiR-203plants were inoculated with *Magnaphorte oryzae spores*. Expression analyses were

carried out by RT-qPCR 48 and 72 hours after inoculation. Mock-inoculated and inoculated plants are represented by black and grey bars, respectively. As expected defense marker genes have a less or similar respond to infection in transgenic lines plants compared to control plants. Statistical significance was determined by ANOVA (*, P ≤0.05; **, P ≤0.01; ***, P ≤0.001). Histograms show the mean ± SD.

Material and methods

Plant material and fungal material

Rice plants (*Oryza sativa* L. cv. Nipponbare) were grown at 28 ± 25ºC with a 16h/8h light/dark cycle. The *M. oryzae* isolate Guy11 (kindly provided by Dr. A. Sesma, Madrid) as used for blast disease resistance assays (Sesma and Osbourn, 2004). The fungus was grown for two weeks at 28°C in oatmeal agar (30mg/mL cloranfenicol) in Petri dishes. Spores were collected in sterile water from fungal mycelium, filtered with miracloth, and adjusted to the appropriate concentration with a Bürker counting chamber.

Cloning of miRNA precursors and transient expression assays in *Nicotiana benthamiana* leaves.

The DNA fragment encompassing the entire precursor sequence with a plus extension at its 5' and 3' ends was PCR amplified from rice genomic DNA using primers listed in Supplemental Table 1. The length of the PCR amplified DNA fragments containing the precursor sequences for each candidate miRNA were: 314 bp, 322 bp, 298 bp, 436 bp, and 251 bp for miR-64, miR75, miR-96, miR98 and miR-203, respectively. The DNA fragment containing the pre-miRNA sequence was cloned into the pCAMBIA1300 expression vector under the control of the maize *Ubiquitin1* promoter (*Ubi1*) and the *nopaline synthase 3*' polyadenylation region (*nos*) to obtain the *pC1300::Ubi1::pre-miRNA::nos* construct (Lacombe et al., 2008). Transient expression assays were carried out in leaves of four-week-old *Nicotiana benthamiana*. For this, the expression vector construct was transferred to the *Agrobacterium tumefaciens* EAH105 strain. As a negative control, the empty vector was used. To avoid transgene-derived production of siRNAs, the *rdr6IR N. benthamiana* line was used in agroinfiltration experiments (Schwach et al., 2005). In addition to RDR6, the agroinfiltrated tissue was also deficient in RDR1 (*N. benthamiana* possesses a naturally-occurring loss-of-function mutation in the *RDR1* gene). Two days after agroinfiltration, the leaf tissues were analyzed by Northern blot using oligonucleotide probes complementary to each sequenced of small RNA (Supplemental Table 1).

Rice transformation and molecular characterization of transgenic lines.

Transgenic rice lines expressing a miRNA precursor of interes were produced by *Agrobacterium*-mediated transformation (*A. tumefaciens* EHA105 strain) of embryogenic calli derived from mature embryos (Sallaud et al., 2003). The parent pCAMBIA 1300 vector already contained the *hptII* (*hygromycinphosphotransferase*) gene encoding hygromycin resistance in the T-DNA region. Transgene integration and integrity was confirmed by PCR of primary T0 transformants using PCR primers located in the promoter and precursors regions (Supplemental Table 1). The transgene copy number was estimated by qPCR in homozygous transgenic rice lines, using the single copy *SPS* (*sucrose phosphate synthase*) gene for normalization (Yang et al., 2005).

Blast resistance assays.

Resistance to infection by the rice blast fungus *M. oryzae* strain *Guy11* was determined on soil-grown plants at the three-leaf stage. The plants were spray inoculated with a spore suspension at the desired concentration and maintained in a chamber under high humidity conditions. The progress of disease symptoms was followed with time. At 7 days post-inoculation, the second leaf of each plant was collected and digital images were taken. Lesion areas were quantified by Image Analysis Software, Assess 2.0, for plant disease quantification (Lamari, 2008). ANOVA test was used to evaluate significant differences in leaf areas affected by blast lesions.

Quantification of fungal DNA was carried out by real-time PCR using specific primers for the *Magnaporthe oryzae* 28S and normalized to the *O. sativa UBIQUITIN1* (*Os06g46770*) gene as an internal control (Qui and Yang, 2002). PCR primers are listed in Supplemental Table 1. Three independent infection experiments were carried out. In each experiment, three independent miRNA overexpressor lines and three independent empty vector lines were assayed (15 plants per line). Azygous (segregated progenies of primary transformants) and wild type plants were included in infection experiments.

Gene expression analysis.

Total RNA was extracted from leaves using TRIzol reagent (In vitrogen). The low molecular weight fraction was obtained from total RNA by PEG8000/NaCl precipitation. For Northern blot analysis, small RNAs were fractionated in a 17.5% denaturing polyacrylamide gel containing 8M urea, transferred to nylon membranes and probed with (γ^{32}P) ATP-labelled oligonucleotides (Supplemental Table 1). Hybridization signals were

detected using a Phosphorimager (Bio-Rad). Synthetic RNA oligonucleotides were loaded as size markers.

RT-PCR was carried out to examine the accumulation of transcripts for miRNA precursors. Reverse transcription reactions were performed using DNase-treated total RNA (1 µg), reverse transcriptase (Applied Biosystems) and oligo-dT_{18} (Sigma, Aldrich). PCR primers were designed to encompass the entire stem loop structure of the corresponding precursor (Supplemental Table 1). In the case of miR-203, nested PCR reactions were carried out for detection of transcripts for this precursor. The PCR products were verified by nucleotide sequencing. Controls without adding the reverse transcriptase enzyme were systematically included in RT-PCR reactions to check for the absence of genomic DNA.

Quantitative reverse transcription PCR (RT-qPCR) 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) (Supplemental Table 1). The *UBIQUITIN5* (*Os01g22490*) gene was used as the internal control for normalization. Three independent biological replicates were analyzed. ANOVA tests were used to evaluate differences in gene expression.

Bioinformatic analysis

Computational prediction of miRNA precursor structures was carried out using the RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). To search for orthologous sequences in the genome of plant species for miRNA sequence homology was carried out by BLASTN. For this, genomic sequences spanning the putative mature miRNA sequences were extracted from the National Center for Biotechnology Information, NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Target prediction for rice miRNAs was performed using the psRNATarget program (http://plantgrn.noble.org/psRNATarget/) using the *O. sativa* cDNA dataset by The Institute for Genomic Research (TIGR).

Discussion

Plant miRNAs are key regulators of gene expression in a wide range of developmental processes, including organ polarity and morphogenesis, flowering, shoot and root development, and hormone signaling. There are also reports indicating that miRNAs are involved in the plant response to abiotic stress, oxidative stress and nutrient deficiency. In rice, the relevance of distinct miRNAs in controlling traits of agronomic importance, such as tiller growth, early flowering, panicle and grain production, is well demonstrated (Miura et al., 2010; Wang et al., 2012; Zhang et al., 2013). Less is known, however, on the involvement of miRNAs in plant-microbe interactions, particularly in the rice/*M. oryzae* interaction. In rice, a total of 592 precursors and 713 mature miRNAs are registered in miRBase (miRbase release 21), but the biological function for most of them is still unknown. Thus, the aim of this study was investigate the functional role of miRNA candidates in rice innate immunity.

In addition to expression profiling, high-throughput sequencing has proven to be useful to discover novel miRNAs. Along with this, the characterization of small RNA transcriptomes from rice leaves that had been treated, or not, with *M. oryzae* elicitors revealed the presence of small RNAs representing novel miRNA candidates (Campo et al., 2013). Five of these miRNA candidates have been the object of this study. Table 12 summarizes the results obtained in the study of the 5 miRNA candidates.

	miR-75	miR-64	miR-96	miR-98	miR-203
Detection in rice leaves	miR-75-5p miR-75-3p	miR-75-5p miR-75-3p	miR-75-5p miR-75-3p	miR-75-5p miR-75-3p	miR-75-5p miR-75-3p
DCL dependency	DCL3	DCL3/DCL4	DCL3/DCL4	-	-
Phenotype / blast Overexpression	Resistance	Resistance	Susceptibility	Susceptibility	Susceptibility
Phenotype / blast (CRISPR/Cas9 mutation)	Susceptibility	-	-	-	-
Target gene	*β-1,3-glucanase*	-	-	-	-

Table 12. Summary of results obtained in the study of rice miRNAs. -, not determined

To start, we computationally predicted miRNA stem loop precursor structures containing sequenced small RNAs. The accumulation of small RNA sequences corresponding to miRNA-5p and miRNA-3p species was confirmed by Northern blot analysis. Thus, detection of both small RNAs indicated that all 5 candidate miRNAs might represent previously uncharacterized miRNAs from rice. All of them were found to accumulate at

very a low level in rice leaves, as judged by both the low number of reads found in small RNA sequencing datasets and the large amount of RNA (small RNA fraction) needed for their detection by Northern blot analysis.

It is known that pathogen infection or treatment with elicitors is accompanied by dynamic alterations in the expression of an important number of known miRNAs (Campo et al., 2013; Baldrich et al., 2015). The observed dynamic response in miRNA accumulation (e.g. up- and down-regulation regulation during elicitor treatment) is consistent with the notion that miRNAs serve as modulators of gene expression rather than turning on or off target gene expression. In this work, the expression profile of these miRNAs during pathogen infection was not examined due to the low leel of accumulation for the miRNAs under study. Moreover, from previous studies in our group, we know that Northern blot analysis gives reliable information of differential expression of miRNAs only in those cases in which relatively large differences occur among samples.

Regarding the origin and evolution of miRNAs, it is generally assumed that *MIR* genes are under constant evolution. The frequent birth and death of Arabidopsis *MIR* genes is well documented (Fahlgren et al., 2007; Nozawa et al., 2012). The prevalent model to explain the origin of plant miRNAs is that they arise from inverted duplication of their target genes, which generates a proto-*MIR* gene (Allen et al., 2004; Fahlgren et al., 2007; Axtell and Bowman, 2008). Accumulation of mutations would shape the proto-*MIR* into a young *MIR* gene, and eventually, and ancient *MIR* gene. The accumulation of mutations during evolution of *MIR* genes also leds to a progressive shift in DCL usage from young miRNAs (processed by DCL4 and DCL4) to old “ancient” miRNAs (processed by DCL1) (Vazquez et al., 2008; Cuperus et al., 2011). In this work, we show that the accumulation of miR-75 is clearly reduced in the *dcl3* mutant, whereas miR-64 and miR-96 precursor processing depends on both DCL3 and DCL4 activities. Altogether, these findings indicated that miR-64, miR-75 and miR-96 are high confidence miRNAs. Most probably, these miRNAs are “young”, recently evolved *MIR* genes. Further studies will allow us to infer DCL dependency for precursor processing of miR-98 and miR-203.

The biological interpretation of novel miRNA functioning is limited, unless functional studies in transgenic/mutant plants are carried out. In this work transgenic rice plants overexpressing a miRNA of interest were generated. Disease resistance assays of transgenic rice plants demonstrated that all 5 miRNA candidates are involved in the rice

response to infection by *M. oryzae*. Thus, rice plants constitutively expressing either miR-64 or miR-75 exhibited enhanced resistance to the rice blast fungus *M. oryzae*, indicating that these miRNAs function as positive regulators in the rice defense response to fungal infection. The observed susceptibility to *M. oryzae* infection in rice lines that have been silenced for *MIR-75* expression using the CRISPR/Cas9 system (see Chapter 3 of the present Thesis) further support the involvement of miR-75 and disease resistance. When overexpressing miR-96, miR-98 or miR-203, the transgenic plants showed increased susceptibility to *M. oryzae* infection, suggesting that these miRNAs function as negative regulators in rice immunity. Taking together, miR-64, miR-75, miR-96, miR98 and miR-203, can be considered components of the complex regulatory network controlling the rice response to *M. oryzae* infection.

On the other hand, the prediction of miRNA targets using computational programs based on sequence alignment is widely used to infer the biological function of miRNAs. Different parameters are used for *in silico* prediction, such as alignment score, number of consecutive mismatches, number of G::U wobble pairing (allowance of a G pairing with a U instead of a C), and number of gaps (Xie and Zhang, 2010). miRNA-guided cleavage of target transcripts occurs at its site opposite to the 10^{th} and 11^{th} positions from the 5' end of the miRNA. To accurately validate miRNA target genes, the RNA Ligase-Mediated (RLM)-Rapid Amplification of cDNA Ends (RACE) method is currently used. The method was later on exploited to develop the degradome sequencing technology for high-throughput miRNA target identification in plant species (Aldo-Quaye et al., 2009; German et al., 2009). Taking advantage of the available information on degradome sequencing of rice libraries, cleavage events corresponding to predicted target genes for some of the miRNAs under study were identified (e.g. miR-75 and miR-98). Of interest, a β-1,3-glucanase gene was identified as the target gene for miR-75. The complementary sites for both miR-75-5p and miR-75-3p were identified in the 3' untranslated region of transcripts encoding a β-1,3-glucanase gene from rice. Degradome analysis also revealed a regulation of various mRNA targets by miR-98. Degradome tags were not identified for miR-64, miR-96 and miR-203. Further studies are, however, needed for the identification of target genes for miR-64, miR-96 and miR-2013.

The identification of β-1,3-glucanase as the target gene for miR-75 provides further evidence that this is *bona fide* miRNA involved in disease resistance in rice. Plant β-1,3-glucanases are pathogenesis-related (*PR*) proteins, which belong to the *PR-2* family of

PR proteins and play an important role in plant defense responses to pathogen infection. These enzymes catalyze the cleavage of the β-1,3-glucosidic bonds in β-1,3-glucan, a major structural component of the cell walls of many pathogeni fungi. In addition, β-1,3-glucanases are involved in the production of endogenous oligosaccharides that might elicit the production of other *PR* proteins or low molecular weight antifungal compounds, such as phytoalexins.

Now, it will be of interest to examine the expression of target transcripts identified by degradome sequencing and the corresponding regulatory miRNAs in response to *M. oryzae* infection. Expression analysis of miRNA target genes identified by degradome analysis (as well as of predicted target genes), together 5'-RACE experiments with the transgenic rice lines generated in this work, will be carried out to further demonstrate a miRNA-mediated cleavage of those target genes.

Overall, the information gained in this study reinforce the relevance of miRNA function in plant immunity. At present, most of our knowledge on miRNAs controlling disease resistance comes from studies in Arabidopsis during interaction with the bacterial pathogen *P. syringae*, and limited information is currently available on miRNAs involved in rice immunity. Only 3 miRNA have been functionally characterized in the rice/*M. oryzae* interaction. They are: miR7695, miR160 and miR398. In particular, miR7695 is a rice-specific, recently evolved miRNA that experienced natural and domestication events during rice evolution (Campo et al., 2013). The rice miR7695 was described to negatively regulate the accumulation of an alternatively spliced transcript of the *OsNramp6* (Natural resistance-associated macrophage protein 6) gene, and its overexpression confers resistance to infection by the rice blast fungus *M. oryzae* (Campo et al., 2013). miR160 targets Auxin Response Factors (ARFs) involved in auxin signaling, and auxins are known to play a crucial role in development and control of plant immune responses in both Arabidopsis and rice plants (Navarro et al., 2006; Domingo et al., 2009). Concerning miR398, it targets targets two Cu/Zn superoxide dismutase genes (*CSD1* and *CSD2*) and a copper chaperone for superoxide dismutase, these enzymes acting as scavengers of ROS. Then miR398 is involved in protection against oxidative stress associated to pathogen infection (Sunkar and Zhu, 2004; Jagadeeswaran et al., 2009). Overexpression of miR160a or miR398b in transgenic rice enhances resistance to *M. oryzae* (Li et al., 2014). Although there is still much to learn on miRNAs contributing to disease resistance, this piece of evidence supports the usefulness of *MIR* genes to prevent disease in rice, a

monocotyledonous species that has been adopted as the model cereal for functional genomics.

In summary, results here presented illustrates the potential of using miRNAs for rice protection while providing the basis to investigate miRNA-mediated regulation of gene expression in the response of rice plants to pathogen infection. *M. oryzae* is one of the primary causes of rice losses worldwide. Efforts to identify gene regulation networks in which distinct miRNAs participate might have important implications for the development of novel strategies for rice protection. Knowing that rice has been adopted as the model species in cereal genomics, investigation on miRNA-mediated regulated processes will benefit other cereal research programs.

References

Addo-Quaye C., Eshoo T.W., Bartel D.P., Axtell M.J. 2008. Endogenous siRNA and miRNA targets identified by sequencing of the Arabidopsis degradome. Curr Biol. 18, 758-762.

Agrawala G.K., Rakwalb R., Jwac N-S., Agrawala V.P. 2001. Signalling molecules and blast pathogen attack activates rice *OsPR1a* and *OsPR1b* genes: A model illustrating components participating during defence/stress response. Plant Physiol. and Biochem. 39, 1095–1103.

Allen E., Xie Z., Gustafson A.M., Sung G.H., Spatafora J.W., Carrington J.C. 2004. Evolution of microRNA genes by inverted duplication of target gene sequences in *Arabidopsis thaliana*. Nat. Genet. 36, 1282-1290.

Ambros V., Bartel B., Bartel D.P., Burge C.B., Carrington J.C., Chen X., et al. 2003. A uniform system for microRNA annotation. RNA. 9, 277-279.

Axtell M.J., Bowman J.L. 2008. Evolution of plant microRNAs and their targets. Trends Plant Sci.13, 343-349.

Axtell M.J., Westholm J.O., Lai E.C. 2011. Vive la différence: biogenesis and evolution of microRNAs in plants and animals. Genome Biol. 12:221.

Baldrich P., San Segundo B. 2016. MicroRNAs in Rice Innate Immunity. Rice 9, 1–6.

Baldrich P., Campo S., Wu M-T., Liu T-T., Hsing Y-IC., San Segundo B. 2015. MicroRNA-mediated regulation of gene expression in the response of rice plants to fungal elicitors. RNA Biol. 12, 847–863.

Boccara M., Sarazin A., Thiébeauld O., Jay F., Voinnet O., Navarro L., Colot V. 2014. The Arabidopsis miR472-RDR6 Silencing Pathway Modulates PAMP- and Effector-Triggered Immunity through the Post-transcriptional Control of Disease Resistance Genes. PLoS Pathogens. 10, 1–16.

Boller T., Felix G., 2009. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. Ann. Rev. of plant Biol. 60, 379-406.

Bonnet E., Wuyts J., Rouze P., de Van Peer Y. 2004. Detection of 91 potential conserved plant microRNAs in *Arabidopsis thaliana* and *Oryza sativa* identifies important target genes. Proc. Natl. Acad. Sci. 101, 11511–11516.

Campo S., Peris-Peris C., Siré C., Moreno A.B., Donaire L., Zytnicki M., et al. 2013. Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the *Nramp6 (Natural resistance-associated macrophage protein 6)* gene involved in pathogen resistance. New Phytologist. 199, 212–227.

Chapman E.J., Carrington J.C. 2007 Specialization and evolution of endogenous small RNA pathways. Nat. Rev. Genet. 8, 884–896.

Cuperus J.T., Fahlgren N., Carrington J.C. 2011. Evolution and functional diversification of MIRNA genes. Plant Cell. 23, 431–442.

Dai X., Zhao P.X. 2011. psRNATarget: a plant small RNA target analysis server. Nucleic Acids Res. 39:W155-159.

Denancé N., Ranocha P., Oria N., Barlet X., Rivière M-P., Yadeta K.A., et al. 2013. *Arabidopsis* wat1 (walls are thin1)-mediated resistance to the bacterial vascular pathogen, *Ralstonia solanacearum*, is accompanied by cross-regulation of salicylic acid and tryptophan metabolism". Plant J. 73, 225-239.

Domingo C., Andrés F., Tharreau D., Iglesias D.J., Talón M. 2009. Constitutive Expression of *OsGH3.1* Reduces Auxin Content and Enhances Defense Response and Resistance to a Fungal Pathogen in Rice. Mol. Plant-Microbe Interactions. 22, 201–210.

Fahlgren N., Howell M.D.,Kasschau K.D., Chapman E.J., et al. 2007. High-Throughput Sequencing of Arabidopsis microRNAs: Evidence for Frequent Birth and Death of *MIRNA* Genes. PLoS ONE. 2:e219.

German M.A., Luo S., Schroth G., Meyers B.C., Green P.J. 2009. Construction of Parallel Analysis of RNA Ends (PARE) libraries for the study of cleaved miRNA targets and the RNA degradome. Nat Protoc. 4, 356-362.

Glazebrook J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Ann. Rev. Phytopathol. 43, 205-227.

Gupta O.P., Sharma P., Gupta R.K., Sharma I. 2014. Current status on role of miRNAs during plant-fungus interaction. Physiol Mol. Plant. Pathol. 85, 1–7.

Jagadeeswaran G., Saini A., Sunkar R. 2009b. Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. Planta. 229, 1009-1014.

Jirage D., Tootle T.L., Reuber T.L., Frost L.N., Feys B.J., Parker J.E., et al. 1999. *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. Proc. Natl. Acad. Sci. USA. 96, 13583-13588.

Jones J.D.G., Dangl J.L. 2006. The plant immune system. Nature. 444, 323–329.

Jones-Rhoades M.W. 2012. Conservation and divergence in plant microRNAs. Plant Mol. Biol. 80, 3-16.

Jones-Rhoades M.W., Bartel D.P., Bartel B. 2006. MicroRNAs an their regulatory roles in plants. Ann. Rev. of Plant Biol. 57, 19–53.

Katiyar-Agarwal S., Jin H. 2010. Role of Small RNAs in Host-Microbe Interactions. Ann.Rev. of phytopatho. 48, 225–246.

Lacombe S., Nagasaki H., Santi C., Duval D., Piegu B., Bangratz M., et al. 2008. Identification of precursor transcripts for 6 novel miRNAs expands the diversity on the genomic organization and expression of miRNA genes in rice. BMC Plant Biol. 8: 123.

Lamari L. 2008. ASSESS 2.0 Image Analysis Software for Plant Disease Quantification. American Phytopathol. Soc. St. Paul.

Lee H.J., Park Y.J., Kwak K.J., Kim D., Park J.H., Lim J.Y., et al. 2015. MicroRNA844-guided Downregulation of *Cytidinephosphate Diacylglycerol Synthase3* (*CDS3*) mRNA Affects the Response of *Arabidopsis thaliana* to Bacteria and Fungi. Mol Plant-Microbe Interact. 28, 892-900.

Li Y., Lu Y.G., Shi Y., Wu L., Xu Y.J., Huang F., et al. 2014. Multiple Rice MicroRNAs Are Involved in Immunity against the Blast Fungus *Magnaporthe oryzae*. Plant Physiol. 164, 1077–1092.

Li Y., Zhang Q., Zhang J., Wu L., Qi Y., Zhou J.M. 2010. Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. Plant Physiol. 152, 2222-2231.

Liu B., Chen Z., Song X., Liu C., Cui X., Zhao X., et al. 2007. *Oryza sativa* dicer-like4 reveals a key role for small interfering RNA silencing in plant development. Plant Cell. 19, 2705–2718.

Liu B., Li P., Li X., Liu C., Cao S., Chu C., Cao X. 2005. Loss of function of OsDCL1 affects microRNA accumulation and causes developmental defects in rice. Plant Physiol. 139, 296–305.

Llave C., Xie Z., Kasschau K.D., Carrington J.C. 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Sci. 297:2053-2056.

Mackey D., Belkhadir Y., Alonso J.M., Ecker J.R., Dangl J.L. 2003. Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates *RPS2*-mediated resistance. Cell. 112, 379-389.

Mallory A., Reinhart B., Jones-Rhoades M., Tang G., Zamore P.D., Barton M.K., Bartel D.P. 2004. MicroRNA control of *PHABULOSA* in leaf development: importance of pairing to the microRNA 5' region. EMBO J. 23, 3356-3364.

Miura K., Ikeda M., Matsubara A., Song X., Ito M., Asano K., et al. 2010 OsSPL14 promotes panicle branching and higher grain productivity in rice. Nat. Genet. 42, 545–549.

Murray M.G., Thompson W.F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8, 4321-4326.

Navarro L., Dunoyer P., Jay F., Arnold B., Dharmasiri N., Estelle M., et al. 2006. A Plant miRNA contributes to Antibacterial Resistance by Repressing Auxin Signaling. Science. 21, 436–439.

Niu D., Lii Y.E., Chellappan P., Lei L., Peralta K., Jiang C., et al. 2016. miRNA863-3p sequentially targets negative immune regulator ARLPKs and positive regulator SERRATE upon bacterial infection. Nat. Commun. 7:11324.

Nozawa M., Miura S., Nei M. 2012. Origins and evolution of microRNA genes in plant species. Genome Biol. Evol. 4, 230-239.

Palatnik J.F., Allen E., Wu X., Schommer C., Schwab R., Carrington J.C., Weigel D. 2003. Control of leaf morphogenesis by microRNAs. Nature. 425, 257-263.

Park Y.J., Lee H.J., Kwak K.J., Lee K., Hong S.W., Kang H. 2014. microRNA400-guided cleavage of pentatricopeptide repeat protein mRNAs renders *Arabidopsis thaliana* more susceptible to pathogenic bacteria and fungi. Plant and Cell Physiol. 55, 1660–1668.

Pumplin N., Voinnet O. 2013. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. Nat Rev. Microbiol. 11, 745-760.

Qi M., Yang Y. 2002. Quantification of *Magnaporthe grisea* During Infection of Rice Plants Using Real-Time Polymerase Chain Reaction and Northern Blot/Phosphoimaging Analyses. Phytopathol. 92, 870-876.

Rajagopalan R., Vaucheret H., Trejo J., Bartel D. 2006. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Genes & Develop. 20, 3407–3425.

Rhoades M.W., Reinhart B.J., Lim L.P., Burge C.B., Bartel B., Bartel D.P. 2002. Prediction of plant microRNA targets. Cell. 23, :513-520.

Robert-Seilaniantz A., Grant M., Jones J.D. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Ann. Rev. Phytopathol. 49, 317-343.

Rogers K., Chen X. 2013. Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell. 25, 2383–2399.

Sablok G., Srivastva A.K., Suprasanna P., Baev V. Ralph P.J. 2015. isomiRs: Increasing Evidences of isomiRs Complexity in Plant Stress Functional Biology. Front. Plant Sci. 6:949.

Sallaud C., Meynard D., van Boxtel J., Gay C., Bès M., Brizard J.P. 2003. Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. Theor. Appl. Genet. 106, 1396-1408.

Schwach F., Vaistij F.E., Jones L., Baulcombe D.C. 2005. An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. Plant Physiol. 138, 1842–1852.

Seo J.K., Wu J., Lii Y., Li Y., Jin H. 2013. Contribution of small RNA pathway components in plant immunity. Mol. Plant Microbe Interact. 26, 617-625.

Sesma A., Osbourn A.E. 2004. The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. Nature. 431:582.

Song X., Li P., Zhai J., Zhou M., Ma L., Liu B., et al., 2012. Roles of DCL4 and DCL3b in rice phased small RNA biogenesis. Plant J. 69, 462-474.

Soto-Suárez M., Baldrich P., Weigel D., Rubio-Somoza I., San Segundo B. 2017. The Arabidopsis miR396 mediates pathogen-associated molecular pattern-triggered immune responses against fungal pathogens. Scientific Reports. In press.

Staiger D., Korneli C., Lummer M., Navarro L. 2013. Emerging role for RNA-based regulation in plant immunity. New Phytol. 197, 394-404.

Sunkar R., Zhu J.K. 2004. Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. Plant Cell. 16, 2001-2019.

Van Loon L.C., Van Strien E.A. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of *PR-1* proteins. Physiol. Mol. Plant 55, 85–97.

Vazquez F., Hohn T. 2013. Biogenesis and Biological Activity of Secondary siRNAs in Plants. Scientifica. 2013:12.

Vazquez F., Blevins T., Ailhas J., Boller T., Meins F. Jr. 2008. Evolution of Arabidopsis *MIR* genes generates novel microRNA classes. Nucleic Acids Res. 36, 6429–6438.

Wang S., Wu K., Yuan Q., Liu X., Liu Z., Lin X., et al. 2012. Control of grain size, shape and quality by *OsSPL16* in rice. Nat. Genet. 44, 950–954.

Wu L., Zhou H., Zhang Q., Zhang J., Ni F., Liu C., Qi Y. 2010. DNA Methylation Mediated by a MicroRNA Pathway. Mol. Cell. 38, 465–475.

Xie F., Zhang B. 2010. Target-align: a tool for plant microRNA target identification. Bioinformatics. 26, 3002-3003.

Yang L., Ding J., Zhang C., Jia J., Weng H., Liu W., Zhang D. 2005. Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR. Plant Cell Rep. 23, 759-763.

Zhang B., Pan X., Cannon C.H., Cobb G.P., Anderson T.A. 2006. Conservation and divergence of plant microRNA genes. Plant J. 46, 243-259.

Zhang X., Zhao H., Gao S., Wang W.C., Katiyar-Agarwal S., Huang H.D., et al. 2011. Arabidopsis Argonaute 2 regulates innate immunity via miRNA393(∗)-mediated silencing of a Golgi-localized SNARE gene, MEMB12. Mol. Cell. 42, 356-366.

Zhang Y-C., Yu Y., Wang C-Y., Li Z-Y., Liu Q.,Xu J., et al. 2013. Overexpression of microRNA OsmiR397 improves rice yield by increasing grain size and promoting panicle branching. Nat. Biotechnol. 3, 848–852.

Name	Accesion number	Sequence (5'-3')	Use
pre-miR-64 Fw	-	CGCGGATCCCTGTGAGCATTGCTTCCCAAACTG	Construct preparation
pre-miR-64 Rv		CGCCCCGGGCAATGCACCCAAACTACATATCAC	
pre-miR-75 Fw	-	CGCGGATCCAGTGTGTATATTTGGACGGATGC	Construct preparation
pre-miR-75 Rv		CGCCCCGGGAACTGACCACATGACAAGGGTAT	
pre-miR-96 Fw	-	CGCGGATCCAGCTCAGAGCATGCTAACCTAGA	Construct preparation
pre-miR-96 Rv		CGCCCCGGGTTTGCCTACCTTGGCCTACA	
pre-miR-98Fw	-	CGCGGATCCGGGGGCAAGAACAAAGCCATTG	Construct preparation
pre-miR-98 Rv		CGCCCCGGGCCAGCCATTTGATCCCAAAGAC	
pre-miR-203 Fw	-	CGCGGATCCGCAAGCATATCACTACCCAAACA	Construct preparation
pre-miR-203 Rv		CGCCCCGGGGCAACCCCTTGGATAGTGATAC	
miR-64.1-5p	-	CACACAAAACAAGACAAGCCATTG	Northern Blot (probe)
miR-64-3p		TAATGACTTGGCTCGTTTTGCGTGCC	
miR-75-5p	-	ACAATCAAAGTTGGACACGAAAAT	Northern Blot (probe)
miR-75-3p		TTTTCGTGCCCAACGTTTGACCGTC	
miR-96-5p	-	TCCAAATTTGACTGTCCGTCTT	Northern Blot (probe)
miR-96-3p		TAAGACGGACGGTAAAATGTTGGAC	
miR-98-5p	-	TCCATTTCACAATGTAAGCCATTC	Northern Blot (probe)
miR-98-3p		CTAGAATGACTTACATTGTAAAAC	
miR-203-5p	-	CTGCAACATTAGCGATCTATTTCA	Northern Blot (probe)
miR-203-3p		AAAATAGATCGCTAATGTGGCAGC	
U6 small RNA	RF00026	ATTTGGACCATTTCTCGATTTGT	Northern Blot (probe)
pre-miR-64 Fw	-	GCCAATGGCTTGTCTTGTTT	RT-qPCR
pre-miR-64 Rv		GCGTGCCATCAAAAATTCA	
pre-miR-75 Fw	-	CGTGTCCAACTTTGATTGTCCG	RT-qPCR
pre-miR-75 Rv		TCGTGCCCAACGTTTGACCGT	
pre-miR-96 Fw	-	CGTGTCCAACATTTTACCGTCC	RT-qPCR
pre-miR-96 Rv		TGTCCAAATTTGACTGTCCGTC	
pre-miR-98Fw	-	GTAAGTCATTCTAGCATTTCCCACA	RT-qPCR
pre-miR-98 Rv		CCCTCCATTTCACAATGTAAGCC	
pre-miR-203 Fw	-	GTTTGCTGCCACATTAGCGAT	RT-qPCR
pre-miR-203 Rv		TCAGTTTGCTGCAACATTAGCG	
Ubiquitin5 Fw	*LOC_Os01g22490*	TAAGTGCGGCCTCACCTACG	RT-qPCR
Ubiquitin5 Rv		GGAGCCTACGCCTAAGCCTG	
PR1b Fw	*LOC_Os01g28450.1*	ACTGCAGCCTGATCCACTCC	RT-qPCR
PR1b Rv		AGAGGTTCTCGCCAAGGTTGT	
SPS Fw	U33175	TTGCGCCTGAACGGATAT	qPCR (trasgen copy number)
SPS Rv		CATCCCGAAAAGATCAACCG	
hptII Fw	AF294977	CTATTTCTTTGCCCTCGGACGA	qPCR (trasgen copy number)
hptII Rv		CTTCTACACAGCCATCGGTCC	
Ubiquitin1 Fw	*LOC_Os06g46770*	TTCCCCAATGGAGCTATGGTT	qPCR (fungal biomass)
Ubiquitin1 Rv		AAACGGGACACGACCAAGG	
M.oryzae28S Fw	*AB026819*	TACGAGAGGAACCGCTCATTCAGATAATTA	qPCR (fungal biomass)
M.oryzae28S Rv		TCAGCAGATCGTAACGATAAAGCTACTC	
EF1α Fw	*LOC_Os03g08060*	GTGCTCGACAAGCTCAAGGCCG	PCR (genotyping)
EF1α Rw		GTCTGATGGCCTCTTGGGCTCG	
Ubiquitin1 Fw	*LOC_Os06g46770*	CACCTTGGCTGACTACAACATCCA	PCR (genotyping)
Ubiquitin1 Rv		TGCTTACCAGCAAAGATCAGACGC	

Supplemental Table S1. Oligonucleotides sequences used in this study.

CHAPTER II

Silencing *MIR-75* gene expression in rice using the CRISPR/Cas9 system for genome editing

Abstract

Rice blast caused by the fungus *Magnaporthe oryzae* is one of the most devastating diseases of cultivated rice. Breeding for durable resistance to this fungus is difficult not only because of the high degree of pathogenic variability of *M. oryzae* but also because of the large number of fungal races encountered in the field populations. Strategies based on gain and loss-of function of protein-coding defense-related genes have been successfully applied for protection of rice plants against *M. oryzae*. On the other hand, it is nowadays recognized that microRNAs (miRNAs) play crucial roles in processes associated to plant development and adaptation to biotic and abiotic stresses. miRNAs are small, non-coding RNAs that post-transcriptionally regulate gene expression by degradation or translational inhibition of target genes. Although genome editing has proven to be a powerful tool for the functional validation of components in the plant's immune system, the use of such technologies for miRNA loss-of-function has not been fully explored. Mutant alleles for miRNAs are not easily found in insertional mutant collections due to the small size of *MIR* genes. Here, we used the CRISPR/Cas9 system to generate targeted mutagenesis in the *MIR-75* genomic locus in rice. The CRISPR/Cas9-induced mutations plants consisted of insertion or deletion (InDel) mutations at the region adjacent to the mature miR-75 sequence in the stem-loop of the miR-75 precursor. The miR-75 mutant plants showed a higher susceptibility to infection by the rice blast fungus *M. oryzae* which correlates with the observed phenotype of resistance to infection by this pathogen in rice plants overexpressing miR-75. The development of the CRSIPR/Cas9 technology for silencing *MIR* genes represents a useful tool for enhancing blast resistance as well as for functional studies on miRNA function in rice immunity.

Introduction

Genome editing technologies enable precise modifications of DNA sequences *in vivo* and offer a great potential in crop improvement (Podevin et al., 2013; Voytas and Gao, 2014) Straightforward methodologies for targeted genome editing can have important applications in the functional characterization of plant genes. For many years now, the functional analysis of *MIR* genes has been hampered by the lack of an effective approach to inhibit *MIR* gene expression because of the small size of *MIR* genes. This fact creates difficulties in the production of T-DNA insertional knock-out mutants. Alternative approaches have been developed to repress endogenous *MIR* expression and/or miRNA activity which have been mainly developed in Arabidopsis plants. They include, blocking miRNA activity by the target mimicry technology (Franco-Zorrilla et al., 2007; Todesco et al., 2010), suppression of microRNA accumulation via RNA interference by targeting the primary miRNA transcripts or their promoters (Vaistij et al., 2010), and artificial miRNA-directed silencing of *MIR* genes (Eamens et al., 2010). As a matter of interest, targeted genome engineering has emerged as an alternative to transgenic methods, and has opened new possibilities to mutagenize genomes at specific loci.

The technology of genome editing arises from our knowledge on the infection process used by bacterial plant pathogens as it exploits the knowledge gained on the Transcription activator-like (TAL) effectors of *Xanthomonas oryzae* that are delivered into the host cells via the bacterial type III secretion system (Li et al., 2013). Members of the TAL effector family act as virulence factors of *Xanthomonas* by reprogramming host gene expression, i.e. by transcriptionally activating specific disease-susceptibility host genes, thus aiding in bacterial infection. TAL proteins recognize plant DNA sequences through a central repeat domain consisting of a variable number of ~34 amino acid repeats. The number and order of repeats in a TAL effector determines its specificity (e.g., one repeat determines recognition of one DNA base pair) (Boch et al., 2009). These proteins are of interest in plant research for the relative ease of retargeting them to bind new DNA sequences. An important consequence of the TAL effector code is that artificial effectors with novel repeat orders, and thus novel DNA target preferences, can be constructed. Numerous groups have designed artificial TAL effectors capable of recognizing new DNA sequences in a variety of experimental systems. Based on these properties, TAL effector

nuclease (TALEN) fusion proteins have been developed in which the DNA recognition repeats of native or customized TAL effectors are fused to the DNA cleavage domains of an endonuclease, such as the FokI endonuclease. In this way, TALENs bind and cleave an exact site in a genome (Cermak et al., 2011; Joung and Sander, 2013). The TALEN technology has been used successfully to develop disease-resistant rice plants that do not contain foreign DNA by targeting the rice susceptibility gene *Os11N3* for TALEN-based disruption (Li et al., 2012, 2013).

More recently, a RNA-guided gene-editing technology has been developed based on the clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease 9 system (CRISPR/Cas9) has been developed for genome editing and targeted mutagenesis in plants (Miao et al., 2013; Shan et al., 2013; Belhaj et al., 2015; Lowder et al., 2015; Endo et al., 2016; Paul and Qi 2016; Quétier, 2016). The system includes the Cas9 nuclease and an engineered guide RNA, either single guide RNA (sgRNA) or dual-sgRNA that specifies a targeted nucleic acid sequence. The sgRNA is a chimera of the naturally occurring CRISPR RNA (crRNA), which is complementary to the target DNA sequence, and the trans-activating CRISPR RNA (trcrRNA), which forms a structural bridge between the crRNA and Cas9. Cas9 is an RNA-guided DNA nuclease containing the catalytically active nuclease domains (NHN and RuvC). The sgRNA/Cas9 complex is recruited to the target sequence by the base-pairing between the sgRNA sequence. For successful binding of Cas9, the genomic target sequence must contain the correct Protospacer Adjacent Motif (PAM) sequence immediately following the target sequence. Cas9 will mainly cut 3-4 nucleotides upstream of the PAM sequence. Deletions of genes, and gene replacement, can be also achieved via the simultaneous delivery of a pair of sgRNAs designed to target both of the flanking regions of the gene of interest (Zhao et al., 2016).

Compared with protein-guided technologies, the CRISPR/Cas9 system is much easier to implement, as only short guide RNAs need to be customized to target the gene of interest. Up to now, the CRISPR/Cas9 system has been successfully applied to efficient genome editing in different plant species, including major crops such as rice (Feng et al., 2013; Ikeda et al., 2016), maize (Liang et al., 2014; Svitashev et al., 2015), wheat (Upadhyay et al., 2013; Shan et al., 2014; Wang et al., 2014), shorgum (Jiang et al., 2013), tomato (Brooks et al., 2014; Ito et al., 2015), soybean (Jacobs et al., 2015; Li et al., 2015) and potato (Wang et al., 2015). Due to the high efficiency of the CRISPR/Cas9

system, it can introduce bi-allelic or homozygous mutations directly in the first generation of rice transformants (Zhang et al., 2014; Bortesi and Fischer, 2015). The CRISPR-based technique makes the introduction of modifications in a plant genome possible, and these are indistinguishable from those introduced by conventional mutagenesis (i.e. chemical or physical mutagenesis).

The application of genome editing technologies for plant disease resistance is a fast growing field of research (Andolfo et al., 2016). For instance, the TALEN and CRISPR/Cas9 technologies were both used to target *MLO* (*mildew-resistance locus O*) in wheat to produce plants resistant to powdery mildew disease (Wang et al., 2014). The usefulness of the CRISPR/Cas9 technology in rice protection is illustrated by results obtained in rice blast resistance through targeted mutagenesis of an *ERF* transcription factor gene (*OsERF922*) (Wang et al., 2016). The CRISPR/Cas9 technology might have important applications for protection of rice plants against pathogen infection by creating targeted mutagenesis in *MIR* genes, an aspect that still remains unexplored. In this study, the CRIPR/Cas9 system has been successfully applied to direct *MIR-75* silencing, a novel miRNA from rice involved in resistance to infection by the blast fungus *Magnaporthe oryzae*.

Results and Discussion

CRISPR/Cas9 design and rice transformation

In recent years, tools to streamline the construction of CRISPR/Cas9 plasmids for *Agrobacterium*-mediated transformation in plants have rapidly emerged (Paul and Qi, 2016). In this study, vectors described by Miao et al (2013), namely the *pH-Ubi-cas9-7* and *pOs-sgRNA* plasmids, were used to produce CRIPR/Cas9 transgenic lines silenced for *MIR-75* expression. These vectors were kindly provided by Dr. Li-Jia Qu (Miao et al., 2013).

To design a CRISPR/Cas9 vector targeting the rice *MIR-75* locus, a 20-nucleotide sequence (spacer) located in the miR-75 precursor region was chosen as the target site. The predicted Cas9 cleavage site was close to the mature miR-75-5p sequence (Figure 1A). To minimize off-target effects, the CRISPR-P software (http://cbi.hzau.edu.cn/crispr/) (Lei et al., 2014) was used to identify sgRNA sequences in the genome region encompassing the miRNA precursor and/or the promoter region. For preparation of the plant expression vector, the spacer DNA fragment was initially cloned into the entry vector (*pOs-sgRNA*). Then, the DNA sequence containing the spacer and single guide RNA (sgRNA) under de control of the maize *Ubi3* promoter was cloned into the *pH-Ubi-cas9-7* plasmid (which contains the Cas9) to obtain the *pH-Ubi-cas9-7-premiR-75* construct (Figure 1B).

A

B

pH-Ubi-cas9-7-premiR-75

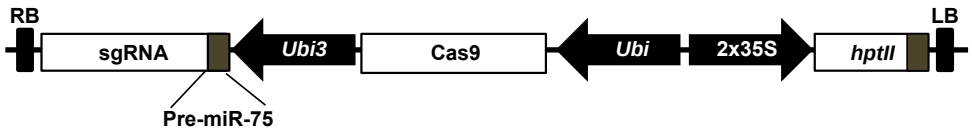


Figure 1. CRISPR/Cas9-induced *MIR-75* gene modification in rice. (A) Schematic of the miR-75 target site for CRISPR/Cas9-induced *MIR-75* gene modification in rice. The target site nucleotides are shown in bold letters. The protospacer adjacent motif (PAM) site is highlighted in orange color. The mature miR-75-5p sequence is underlined. **(B)** Schematic diagram of the T-DNA in the *pH-Ubi-cas9-7-premiR-75* construct. sgRNA (Single guided RNA); *Ubi3* (*Ubiquitin3*); Cas9 (CRISPR associated protein 9); 35S (Cauliflower mosaic virus 35S promoter); *hptII* (*hygromycin phosphotransferase II*).

Generation of transgenic rice plants with mutations in *MIR-75*

Transgenic rice (*O. sativa* cv Nipponbare) lines were produced by *Agrobacterium*-mediated transformation. To confirm the specificity and efficiency of DNA cutting, the targeted region was PCR amplified from genomic DNA (Figure 2A) and sequenced. Four independently generated hygromycin resistant lines were analyzed for mutations at the target site. PCR and DNA sequencing of the target-containing amplicons showed that 2 lines contained mutations in the target region (lines 13.1 and 13.3) (Figure 2A). Particularly, the line 13.1 had one nucleotide insertion, whereas line 13.3 had a deletion of 22 nucleotides (Figure 2A). PCR analysis using primers designed to specifically amplify *Cas9* and *hptII* sequences we confirmed the integration of these genes in the genome of the mutant lines (results not shown).

The progeny of the two parental lines (13.1 and 13.3) was genotyped by PCR and DNA sequencing (T1 plants). The allelic mutation in line 13.3 was transmitted to the T1 generation, and the 6 plants derived from line 13.1 had the same allelic mutation than the parental plant. This finding indicated that the T0 mutant plant harboring the 1-nt insertion in the miR-75 precursor structure was homozygous for the mutation, and that the bi-allelic mutation in the T0 plant was transmitted to the T1 generarion.

The progeny from the 13.3 mutant lines was more diverse. Three out of the 6 progeny plants examined, either maintained the 22-nt deletion (13.3-5 and 13.3-6) or had a 3-nt deletion (13.3-2) (Figure 2B). Regarding line 13.4, this line was found to be heterozygous for the CRISPR/Cas9-induced mutation, with the 22-nt deletion and the 3-nt deletion (Figure 2B).

In the literature it has been reported that mutations induced by CRISPR/Cas9 in plants are mainly short deletions (10 bp or less) and that single-base insertions are typically A/T insertions (Bortesi et al., 2016). Longer deletions are less frequently found and single-base substitutions are rare. It is also true that different mutation signatures can be found in rice depending on the target (Miao et al., 2013; Xu et al., 2015). No phenotypical

differences were visible among the T1 mutant lines and wild type plants when grown under controlled greenhouse conditions (Figure 2C).

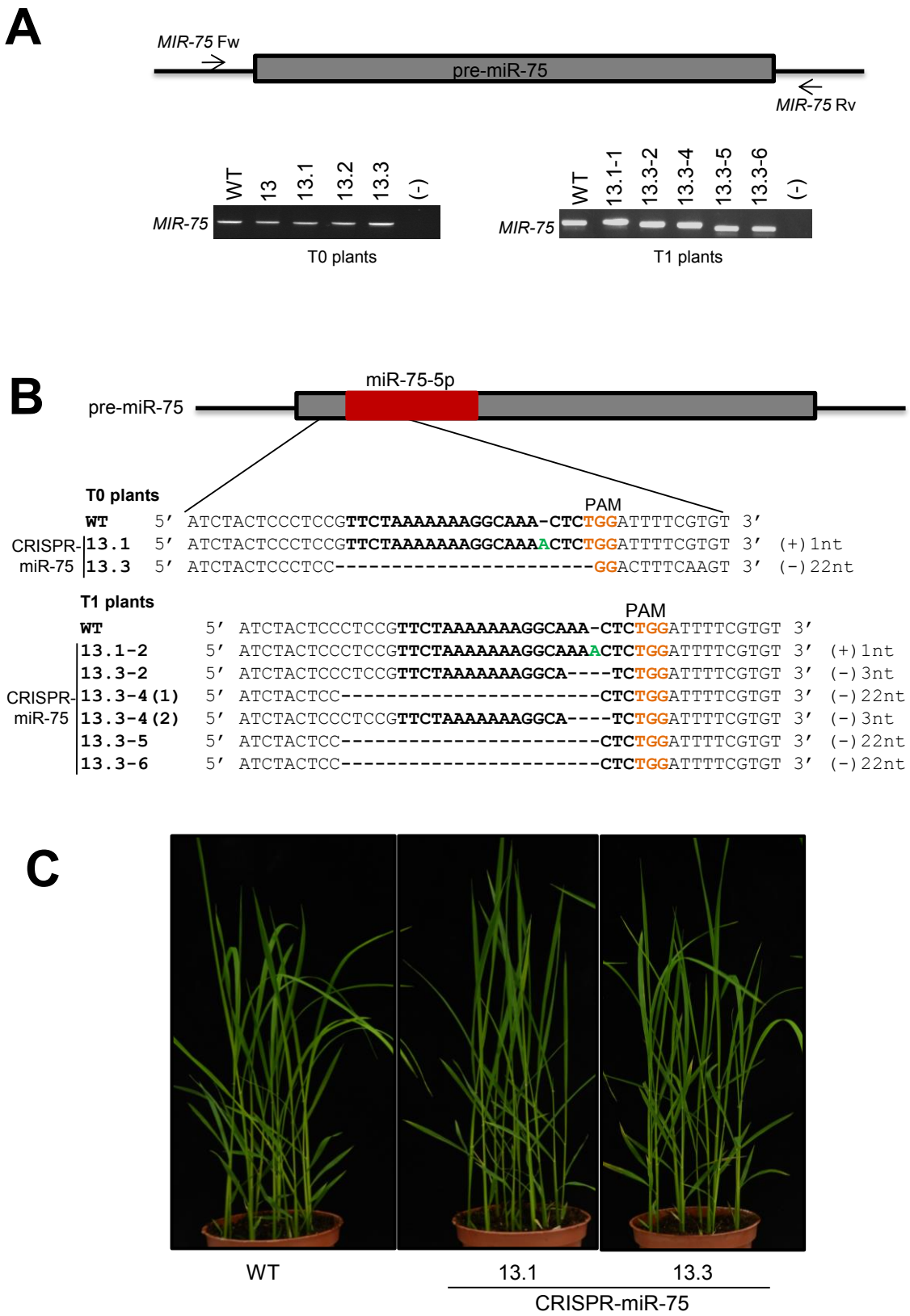


Figure 2. Analysis of CRISPR/Cas9-induced mutations in the *MIR-75* gene. Genotyping of *MIR-75*

mutants. **(A)** PCR-based identification of rice mutants. The *MIR-75* locus was PCR amplified using genomic DNA from the indicated T0 lines using the primers *MIR-75 Fw* and *MIR-75 Rv*. The PCR products were subjected to DNA sequencing. **(B)** Alignment of the nucleotide sequences of the target site (bold letters) in T0 and T1 mutant lines with the control sequence (WT). The protospacer adjacent motif (PAM) site is shown in orange color followed by the mature miR-75-5p sequence. In green, nucleotide insertion in line 13.1-2. **(C)** Phenotype of 3 week-old miR-75 mutant plants. No phenotypic differences were observed between WT and CRISPR miR-75 mutant lines.

Deletion mutations at the *MIR-75* locus increases susceptibility to *M. oryzae* infection

The blast resistance phenotype of mutant rice lines created by CRISPR/Cas9 system was examined in T1 plants derived from the 13.1 and 13.3 mutant lines. As control, wild-type plants (*O. sativa* cv Nipponbare) were used. Two independent experiments (15 plants per genotype) were carried out on 1 line derived from the 13.1 mutant (line 13.1-2) and two lines derived from the 13.3 mutant (lines 13.3-2 and 13.3-5). Infection assays revealed that the three mutant lines here assayed (13.1-2, 13.3-2 and 13.3-5) were more susceptible to infection by the rice blast fungus than wild-type plants (Figure 3A). Differences in susceptibility to infection were confirmed by quantification of the lesion area (Figure 3B) and fungal biomass (Figure 3C). The phenotype of susceptibility to *M. oryzae* infection that is observed in the CRISPR/Cas9 plants is in agreement with the resistant phenotype that occurs in miR-75 overexpressor rice plants (see Chapter 1, Figure 5).

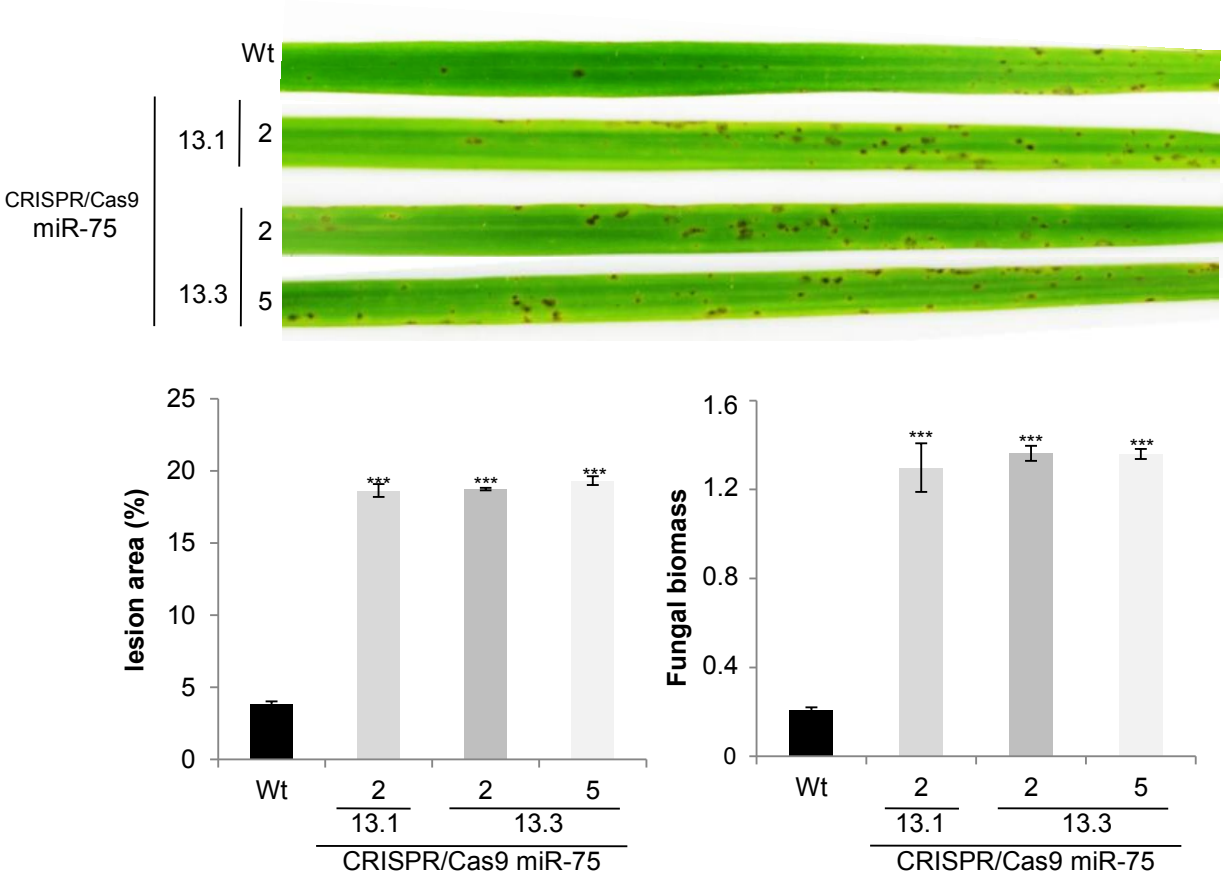


Figure 3. Increased susceptibility of CRISPR miR-75 mutant plants to infection by the rice blast fungus *Magnaphorte oryzae.* (A) Wild-type (*Nipponbare*) and miR-75 mutant lines. The mutated alleles are shown in Figure 2B (line 13.1-2, 1 nucleotide insertion; line 13.3-2, 3 nucleotides deletion; line 13.3-5, 13.3-6 22 nucleotides deletion). Three-week old plants were inoculated with a suspension of *Magnaphorte oryzae* spores (1x10^5spores/ml). Pictures were taken at 7 days after inoculation (7 dpi). **(B)** Quantification of the lesions produced in the leaves at 7dpi by using the Assess 2.0 software for image analysis. **(C)** Quantification of fungal biomass by qPCR using specific primers for the *M. oryzae* 28S and normalized to the *O. sativa UBIQUITIN1* (*Os06g46770*) gene as an internal control.

The secondary stem loop structure of miRNA precursors is critical for processing miRNAs by DCL activities. It is then tempting to hypothesize that mutations generated by the CRISPR/Cas9 system (Indels) on the stem-loop structure of the miR-75 precursor adjacent to the mature miR-75-5p sequence might affect normal processing of the miR-75 precursor, hence, miR-75 functioning. Presumably, deletions found in the mutant plants might affect the precursor secondary structure and processing, which in turn would interrupt accumulation of mature miR-75 sequences. This is particularly evident in mutants having the 22-nt deletion in the precursor structure (Figure 4). Before concluding this, additional studies need to be carried out to demonstrate that indels in the pre-miR-75 structure blocks the production of mature miR-75 sequences in the CRISPR/Cas9 mutant plants (work in progress). It will be also of interest to investigate whether the CRISPR/Cas9-induced mutations are transmitted to successive generations, and whether these plants are free of the *pH-Ubi-cas9-7-premiR-75* construct used for rice transformation. Finally, studies to examine whether off-target mutations are present in the CRISPR/Cas9 mutant rice lines should be carried out.

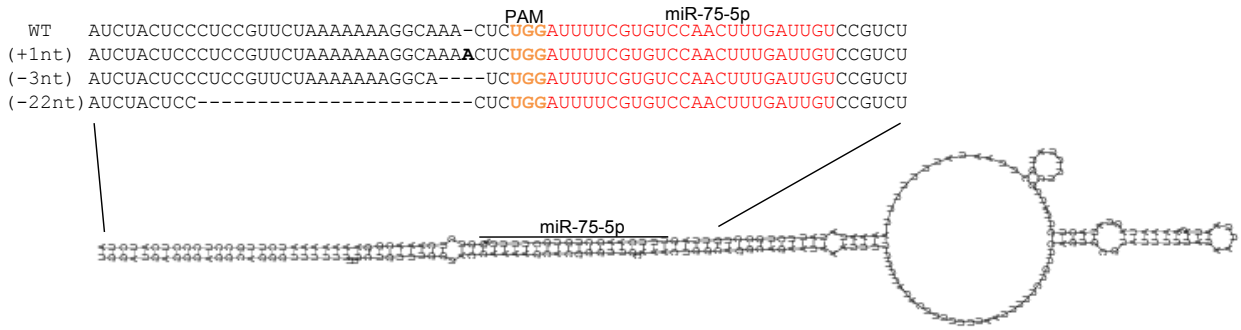


Figure 4. Schematic presentation of the CRISPR/Cas9-induced mutations in the miR-75 precursor structure. The protospacer adjacent motif (PAM) site is shown in orange color and the mature miR-75-5p sequence in red. In bold, nucleotide insertion (line 13.1-2).

Finally, results here presented demonstrated the usefulness of the CRISPR/Cas9 system in editing *MIR* genes in rice plants. The function of only a small number of *MIR* genes has been experimentally demonstrated due, in part, to the lack of an effective approach to silence *MIR* gene expression. Thus, in addition to gain-of-function approaches, it is

particularly important to develop suitable technologies for miRNA silencing when the analysis of miRNA function is pursued. This methodology will broaden and complement our current studies on rice plants overexpressing a miRNA of interest in future research for the functional analysis of miRNAs involved in rice immunity with important implications in rice biotechnology. Using the CRSIPR/Cas9 system for silencing *MIR* genes will eventually strengthen our understanding on miRNA function and miRNA-mediated gene regulatory networks involved in rice immunity.

Material and methods

Vector construction

The vector used in this work for CRISPR/Cas9-induced mutagenesis of *MIR-75* was based on the *pH-Ubi-cas9-7* plasmid in which Cas9 expression is driven by the maize Ubiquitin promoter (*Ubi3*). The vector contains the hygromycin resistance gene (*hptII, hygromycin phosphotransferase II*) as the selectable marker for rice transformation. The *pOs-sgRNA* plasmid was used to introduce the single guide RNA (sgRNA). *The pH-Ubi-cas9-7* and *pOs-sgRNA* plasmids are described by Miao et al., (2013).

Computational prediction of the sgRNA site, target site sequences and prediction of potential off-target sites were performed using the CRISPR-P software (http://cbi.hzau.edu.cn/crispr/) (Lei et al., 2014).

To generate the CRISPR-miR-75 construct, the target site (spacer, 20nt in length) of the miR-75 precursor was PCR amplified from genomic DNA using the following primers: Fw: 5'-GGCATTCTAAAAAAGGCAAACTC-3' and Rv: 5'-AAACGAGTTTGCCTTTTTTTAGAA-3' and cloned into the *BsaI* and *BsmBI* restriction sites of the entry vector (*pOs-sgRNA*). The DNA fragment containing the miR-75 spacer, the sgRNA, and the maize U3 promoter was cloned into the *attR1*and *attR2* recombinant sites of the *pH-Ubi-cas9-7* plasmid (which contains the Cas9). The CRISPR-miR-75 construct was verified by nucleotide sequencing using the primers Fw: 5'-CCGCAAATTAATACAGTACAGGTG-3' and Rv: 5'-AACCTTTAACTGACCACATGACAA-3'.

Rice transformation and molecular characterization mutant CRISPR-miR75 lines.

Trangenic lines were produced by *Agrobacterium*-mediated transformation (*A. tumefaciens* EHA105 strain) of embryogenic calli derived from mature embryos (Sallaud et al., 2003). For identification of mutant transgenic lines, genomic DNA was extracted from hygromycin-resistant T0 plants which was then used for PCR analysis using primers designed to amplify the DNA fragment across the target site in the miR-75 precursor sequence (Fw: 5'-CCGCAAATTAATACAGTACAGGTG-3' and Rv: 5'-AACCTTTAACTGACCACATGACAA-3'). The transgenic lines were also examined by PCR using cas9-specific primers (Fw: 5'-GAGACAGCCGAGGCTACAAG-3' and Rv: 5'-ATGGTCGGGTACTTCTCGTG-3'), and hygromycin-specific primers (Fw: 5'-GCCGATGGTTTCTACAAAGA-3' and Rv: 5'-GAAGAAGATGTTGGCGACCT-3'). All the PCR products were subjected to DNA sequencing.

Plant and fungal material

Rice plants (*Oryza sativa* L. cv. Nipponbare) were grown at 28 ± 25ºC under 16h/8h light/dark photoperiod conditions. The *M. oryzae* isolate *Guy11* (kindly provided by Dr. A. Sesma, Madrid) as used for blast disease resistance assays (Sesma and Osbourn, 2004). The fungus was grown for two weeks at 28°C in oatmeal agar (30mg/mL chloramphenicol). Spores were collected in sterile water from fungal mycelium, filtered with miracloth, and adjusted to the appropriate concentration with a Bürker counting chamber.

Infection assays.

Soil-grown plants at three-leaf-stage were spray-inoculated with a spore suspension of *M. oryzae strain Guy11* ($1x10^5$spores/mL) and maintained in a chamber under high humidity conditions. At 7 days post-inoculation, the second leaf of each plant was collected and digital images were taken. Lesion areas were quantified by Image Analysis Software, Assess 2.0, for plant disease quantification (Lamari, 2008). ANOVA test was used to evaluate significant differences in leaf areas affected by blast lesions.

Quantification of fungal DNA was carried out by real-time PCR using specific primers for the *Magnaporthe oryzae 28S* (Fw: 5'-TACGAGAGGAACCGCTCATTCAGATAATTA-3', Rv: 5'-TCAGCAGATCGTAACGATAAAGCTACTC-3') and normalized to the *O. sativa UBIQUITIN1* (*Os06g46770*) gene as an internal control primers Fw: 5'-TTCCCCAATGGAGCTATGGTT-3', Rv: 5'-AAACGGGACACGACCAAGG-3' (Qui and

Yang, 2002). Two independent infection experiments were carried out (15 plants per line in each experiment).

References

Andolfo G., Iovieno P., Frusciante L., Ercolano M.R. 2016. Genome-Editing Technologies for Enhancing Plant Disease Resistance. Front. Plant Sci. 7:1813.

Belhaj K., Chaparro-Garcia A., Kamoun S., Patron N.J., Nekrasov V. 2015. Editing plant genomes with CRISPR/Cas9. Curr. Opin. Biotechnol. 32, 76-84.

Boch J., Scholze H., Schornack S., Landgraf A.,Hahn S., Kay S., et al. 2009. Breaking the Code of DNA Binding Specificity of TAL-Type III Effectors. Sci. 326, 1509-1512.

Bortesi L., Fischer R. 2015. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol. Adv. 33, 41-52.

Bortesi L., Zhu C., Zischewski J., Perez L., Bassié L., Nadi R., et al., 2016. Patterns of CRISPR/Cas9 activity in plants, animals and microbes. Plant Biotechnol. 14, 2203-2216.

Brooks C., Nekrasov V., Lippman Z.B., Van Eck J. 2014. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol. 166, 1292–1297.

Cermak T., Doyle E.L., Christian M., Wang L., Zhang Y., Schmidt C., et al. 2011. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. 39:e82.

Eamens A.L., Agius C., Smith N.A., Waterhouse P.M., Wang M.B. 2010. Efficient silencing of endogenous microRNAs using artificial microRNAs in *Arabidopsis thaliana*. Mol. Plant. 4,157–170.

Endo M., Mikami M., Toki S. 2016. Biallelic Gene Targeting in Rice. Plant Physiol. 170, 667-677.

Feng Z., Zhang B., Ding W., Liu X., Yang D.L., Wei P., et al. 2013. Efficient genome editing in plants using a CRISPR/Cas system. Cell Res. 23, 1229–1232.

Franco-Zorrilla J.M., Valli A., Todesco M., Mateos I., Puga M.I., Rubio-Somoza I., et al. 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. Nat. Genet. 39,1033-1037.

Ikeda T., Tanaka W., Mikami M., Endo M., Hirano H.Y. 2016. Generation of artificial drooping leaf mutants by CRISPR-Cas9 technology in rice. Genes Genet. Syst. 90, 231–235.

Ito Y., Nishizawa-Yokoi A., Endo M., Mikami M., Toki S. 2015. CRISPR/Cas9-mediated mutagenesis of the *RIN* locus that regulates tomato fruit ripening. Biochem. Biophys. Res. Commun. 467, 76–82.

Jacobs T.B., LaFayette P.R., Schmitz R.J., Parrott W.A. 2015. Targeted genome modifications in soybean with CRISPR/Cas9. BMC biotechnol. 15:16.

Jiang W., Zhou H., Bi H., Fromm M., Yang B., Weeks D.P. 2013. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res. 41:e188.

Joung J.K., Sander J.D. 2013. TALENs: a widely applicable technology for targeted genome editing. Nat. Rev. Mol. Cell Biol. 14, 49-55.

Lamari L. 2008. ASSESS 2.0 Image Analysis Software for Plant Disease Quantification. American Phytopathol. Soc. St. Paul.

Lei Y., Lu L., Liu H.Y., Li S., Xing F., Chen L.L. 2014. CRISPR-P: a web tool for synthetic single-guide RNA design of CRISPR-system in plants. Mol. Plant. 7, 1494-1496.

Li T., Huang S., Zhou J., Yang B. 2013. Designer TAL effectors induce disease susceptibility and resistance to Xanthomonas oryzae pv. oryzae in rice. Mol. Plant. 6, 781-789.

Li T., Liu B., Spalding M.H., Weeks D.P., Yang B. 2012. High-efficiency TALEN-based gene editing produces disease-resistant rice. Nat. Biotechnol. 30, 390–392.

Li Z., Liu Z.B., Xing A., Moon B.P., Koellhoffer J.P., Huang L., et al. 2015. Cas9-guide RNA directed genome editing in soybean. Plant Physiol. 169, 960–970.

Liang Z., Zhang K., Chen K., Gao C. 2014. Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. J. Genet. Genomics. 41, 63–68.

Lowder L.G., Zhang D., Baltes N.J., Paul J.W., Tang X., Zheng X., et al. 2015. A CRISPR/Cas9 Toolbox for Multiplexed Plant Genome Editing and Transcriptional Regulation. Plant Physiol. 169, 971-985.

Miao J., Guo D., Zhang J., Huang Q., Qin G., Zhang X., et al. 2013. Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res. 23, 1233-1236.

Paul J.W., Qi Y. 2016. CRISPR/Cas9 for plant genome editing: accomplishments, problems and prospects. Plant Cell Rep. 35, 1417-1427.

Podevin N., Davies H.V., Hartung F., Nogué F., Casacuberta J.M. 2013. Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding. Trends Biotechnol. 31, 375-383.

Qi M., Yang Y. 2002. Quantification of Magnaporthe grisea During Infection of Rice Plants Using Real-Time Polymerase Chain Reaction and Northern Blot/Phosphoimaging Analyses. Phytopathol. 92, 870-876.

Quétier F. 2016. The CRISPR-Cas9 technology: Closer to the ultimate toolkit for targeted genome editing. Plant Sci. 242, 65-76.

Sallaud C., Meynard D., van Boxtel J., Gay C., Bès M., Brizard J.P. 2003. Highly efficient production and characterization of T-DNA plants for rice (Oryza sativa L.) functional genomics. Theor. Appl. Genet. 106, 1396-1408.

Sesma A., Osbourn A.E. 2004. The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. Nature. 431:582.

Shan Q., Wang Y., Li J., Gao C. 2014. Genome editing in rice and wheat using the CRISPR/Cas system. Nat. Protoc. 9, 2395–2410.

Shan Q., Wang Y., Li J., Zhang Y., Chen K., Liang Z., et al. 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. 31, 686-688.

Svitashev S., Young J.K., Schwartz C., Gao H., Falco S.C., Cigan A.M. 2015. Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. Plant Physiol. 169, 931–945.

Todesco M., Rubio-Somoza I., Paz-Ares J., Weigel D. 2010. A Collection of Target Mimics for Comprehensive Analysis of MicroRNA Function in *Arabidopsis thaliana*. PLoS Genet. 6:e1001031.

Upadhyay S.K., Kumar J., Alok A., Tuli R. 2013. RNA-guided genome editing for target gene mutations in wheat. G3. 3, 2233–2238.

Vaistij F.E., Elias L., George G.L., Jones L. 2010. Suppression of microRNA accumulation via RNA interference in *Arabidopsis thaliana*. Plant Mol. Biol. 73, 391–397.

Voytas D.F., Gao C. 2014. Precision genome engineering and agriculture: opportunities and regulatory challenges. PLoS Biol. 10;12:e1001877.

Wang F., Wang C., Liu P., Lei C., Hao W., Gao Y., et al. 2016. Enhanced Rice Blast Resistance by CRISPR/Cas9-Targeted Mutagenesis of the ERF Transcription Factor Gene *OsERF922*. PLoS One. 11:e0154027.

Wang S., Zhang S., Wang W., Xiong X., Meng F., Cui X. 2015. Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. Plant Cell Rep. 34, 1473–1476.

Wang Y., Cheng X., Shan Q., Zhang Y., Liu J., Gao C., et al. 2014. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat. Biotechnol. 32, 947–951.

Xu R-F., Li H., Qin R-Y., Li J., Qiu C-H., Yang Y-C., et al. 2015. Generation of inheritable and “transgene clean” targeted genome-modified rice in later generations using the CRISPR/Cas9 system. Scientific Rep. 5:11491.

Zhang H., Zhang J., Wei P., Zhang B., Gou F., Feng Z., et al. 2014. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol. J.12, 797-807.

Zhao Y., Zhang C., Liu W., Gao W., Liu C., Song G., et al. 2016. An alternative strategy for targeted gene replacement in plants using a dual-sgRNA/Cas9 design. Scientific Rep. 6:23890.

CHAPTER III

The microRNA miR858 is involved in the immune response of Arabidopsis plants to fungal pathogens

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

Abstract

microRNAs (miRNAs) are a class of short endogenous non-coding small RNA molecules acting as post-transcriptional regulators of gene expression in eukaryotes. Although a large number of miRNAs are known to be regulated during pathogen infection in plants, the biological role of the majority of these pathogen-regulated miRNAs has not been determined. Here, miR858 was investigated for its role in Arabidopsis immunity. The Arabidopsis miR858 targets *MYB* transcription factor genes that function as transcriptional activators of genes involved in flavonoid biosynthesis (*AtMYB11*, *AtMYB12* and *AtMYB111*). We show that miR858 overexpression enhances susceptibility to pathogen infection. By contrast, inactivation of miR858 activity by target mimics (*MIM858* plants), and concomitant up-regulation of miR858 target genes, results in enhanced resistance to infection by necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Fusarium oxysporum*, *Colletotrichum higginsianum*) fungal pathogens. Stronger induction of ethylene regulated defense responses occurs in the fungal-infected *MIM858* plants compared to wild type plants, indicating that miR858 functions as a negative regulator of Arabidopsis immunity. Infection by *P. cucumerina* as well as treatment with elicitors obtained from this fungus results in down-regulation of *MIR858* expression, thus, supporting a role for miR858 in regulating PAMP (Pathogen-associated molecular patterns)-triggered immunity (PTI). Resistance to pathogen infection in *MIM858* plants is associated to flavonoid accumulation in leaves of *MIM858* plants, but not to lignin accumulation. The antifungal activity of phenylpropanoid compounds, including flavonoids, is presented. These findings provide new insights into the relevant role of miR858 in Arabidopsis immunity through the regulation of phenylpropanoid biosynthesis, while demonstrating that suppression of miR858 activity is an effective approach to improve disease resistance in plants.

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; or microbe-associated molecular patterns, MAMPs), 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. Among others, PTI components include production of reactive oxygen species (ROS), reinforcement of 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). PTI and ETI trigger qualitatively similar, but quantitatively and kinetically different transcriptional reprogramming in the host (Tao et al., 2003). Treatment with microbial elicitors triggers the same responses that are observed in infected tissues (Boller and Felix, 2009). The essential role of the phytohormones salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) in resistance to pathogens is also well established (Glazebrook, 2005; Denancé 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 (Penninckx et al., 1999; Thomma et al., 1998, 1999; Glazebrook, 2005; Song et al., 2014). Central to the activation of a successful plant defense response is the ability to modulate gene expression at both the transcriptional and post-transcripional levels.

microRNAs (miRNAs) are a class of small noncoding 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; Chiou et al., 2006; Chen, 2009; Rubio-Somoza and Weigel, 2011; Jeong and Green, 2013). Increasing evidence also supports that miRNAs are key regulators of plant immunity (Katiyar-Agarwal and Jin, 2010; Seo et al., 2013; Staiger et al., 2013; Weiberg et al., 2014; Yang and Huang, 2014: Borges and Martienssen, 2015; Baldrich and San Segundo, 2016; Huang et al., 2016; Kuan et al., 2016). The first evidence for miRNAs affecting pathogen defense came from *A. thaliana*, where a fragment of bacterial flagellin flg22 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. It is also true that immune responses against fungal and bacterial pathogens have been traditionally considered as protein-based defense mechanisms that are regulated at the transcriptional level, largely independent from the RNA-based mechanisms that typically operate in antiviral defense. Our current knowledge on plant miRNAs involved in disease resistance comes mainly from studies in the interaction of Arabidopsis plants with the bacterial pathogen *Pseudomonas syringae*, or treatment with the *P. syringae* flg22 elicitor, and less is known about miRNAs mediating resistance against fungal pathogens.

The general phenylpropanoid pathway metabolism generates an enormous array of secondary metabolites, and for some of then a function has been described (Vogt, 2010; König et al., 2014 Mouradov and Spangenberg, 2014). Specifically, the phenylpropanoid pathway is required for the biosynthesis of flavonoids and monolignols, the building blocks of lignin. 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 phenylpropanoid pathway. Flavonoids have been associated with a variety of biological functions, including UV protection, flower coloring, auxin transport inhibition and plant defense, among others (Buer et al., 2010; Falcone-Ferreyra et al., 2012). Distinct members of the *MYB* (*V-myb myeloblastosis viral oncogene homolog*) family of

transcription factors are known to be key regulators of flavonoid biosynthesis. In *Arabidopsis thaliana*, *AtMYB11*, *AtMYB12*, and *AtMYB111* are all independently capable of activating the genes encoding enzymes specifically involved in flavonoid biosynthesis (Mehrtens et al., 2005; Stracke et al., 2007; Liu et al., 2014; Xu et al., 2015). It is also known that miR858 regulates the expression of the flavonol-specific *AtMYB11*, *AtMYB12* and *AtMYB111* genes (Fahlgren et al., 2007; Aldo-Quaye et al., 2008; Dubos et al., 2010; Sharma et al., 2016). Both miR858 and its *MYB* target genes are evolutionarily conserved in dicots (Xia et al., 2012).

In this study, we used gain- and loss-of-function strategies to investigate whether miR858 plays a role in Arabidopsis immunity. The impact of alterations in *MIM858* expression (overexpression and interference with miR858 activity through target mimics) in resistance to infection by necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Fusarium oxysporum* f. sp. *conglutinans*, *Colletotrichum higginsianum*) fungal pathogens was examined. Whereas Arabidopsis plants overexpressing *MIR858* were more susceptible to fungal infection, enhanced resistance to fungal infection was observed in plants in which the activity of miR858 is suppressed by the target mimic strategy (*MIM858* lines). Resistance to pathogen infections in *MIM858* plants is associated to a stronger induction of ET-mediated defense responses and flavonoid accumulation, but not lignin accumulation. The antifungal activity of the flavonols naringenin and kaempferol, and the phenylpropanoid p-coumaric acid is presented. Overall, results here presented support that miR858 functions as a negative regulator of Arabidopsis immunity by controlling flavonoid accumulation.

Results

Overexpression of *MIR858* increases susceptibility to infection by the fungal pathogen *P. cucumerina.*

In *A. thaliana*, miR858 is encoded by two loci, *MIR858A* and *MIR858B*. Mature miRNAs, both miR858a and miR858b, are 21 nucleotide in length and differ in their last nucleotide (http://www.mirbase.org). To investigate whether miR858 plays a role in Arabidopsis immunity, we generated transgenic plants constitutively expressing either the miR858a or the miR858b precursor sequence (hereafter OE miR858a and OE miR858b, respectively). Control Arabidopsis plants carrying the empty vector were also produced. The transgenic Arabidopsis lines expressing a miR858 precursor accumulated higher levels of the corresponding pre- and mature miR858 sequences (Figure 1A). Under the experimental growth conditions (12h light/12h dark photoperiod), the OE miR858 plants showed a small increase in rosette diameter plants compared with wild type plants, but this phenotypic difference was not statistically significant (Supplemental Figure 1).

The OE miR858 plants were tested for resistance to infection by the fungus *P. cucumerina*. The Arabidopsis/*Plectosphaerella cucumerina* pathosystem is a well-established model for studies on basal resistance to necrotrophic fungi (Palm et al., 1995; Llorente et al., 2005; Sanchez-Vallet et al., 2010). 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, OE miR858a and OE miR858b plants displayed enhanced susceptibility to infection by *P. cucumerina* compared to control plants (wild-type, empty vector plants) (Figure 1B). As expected, *lin1* and *agb1.2* plants showed resistance and susceptibility, respectively, to infection by this pathogen (Figure 1B). Trypan blue staining of *P. cucumerina*-inoculated leaves confirmed extensive fungal colonization in OE miR858 plants whereas, under the same experimental conditions, a few hyphae were observed on leaves of control plants (wild-type, empty vector) (Figure 1C). Susceptibility to fungal infection in OE miR858 plants

was corroborated by determining plant survival of *P. cucumerina*-infected plants (Figure 1D). Finally, qPCR analysis confirmed increased levels of *P. cucumerina* DNA in leaves of fungal-infected OE miR858 plants compared to *P. cucumerina*-inoculated control plants (Figure 1E). From these results, it is concluded that overexpression of miR858, either miR858a or miR858b, increases susceptibility to infection by *P. cucumerina* in Arabidopsis.

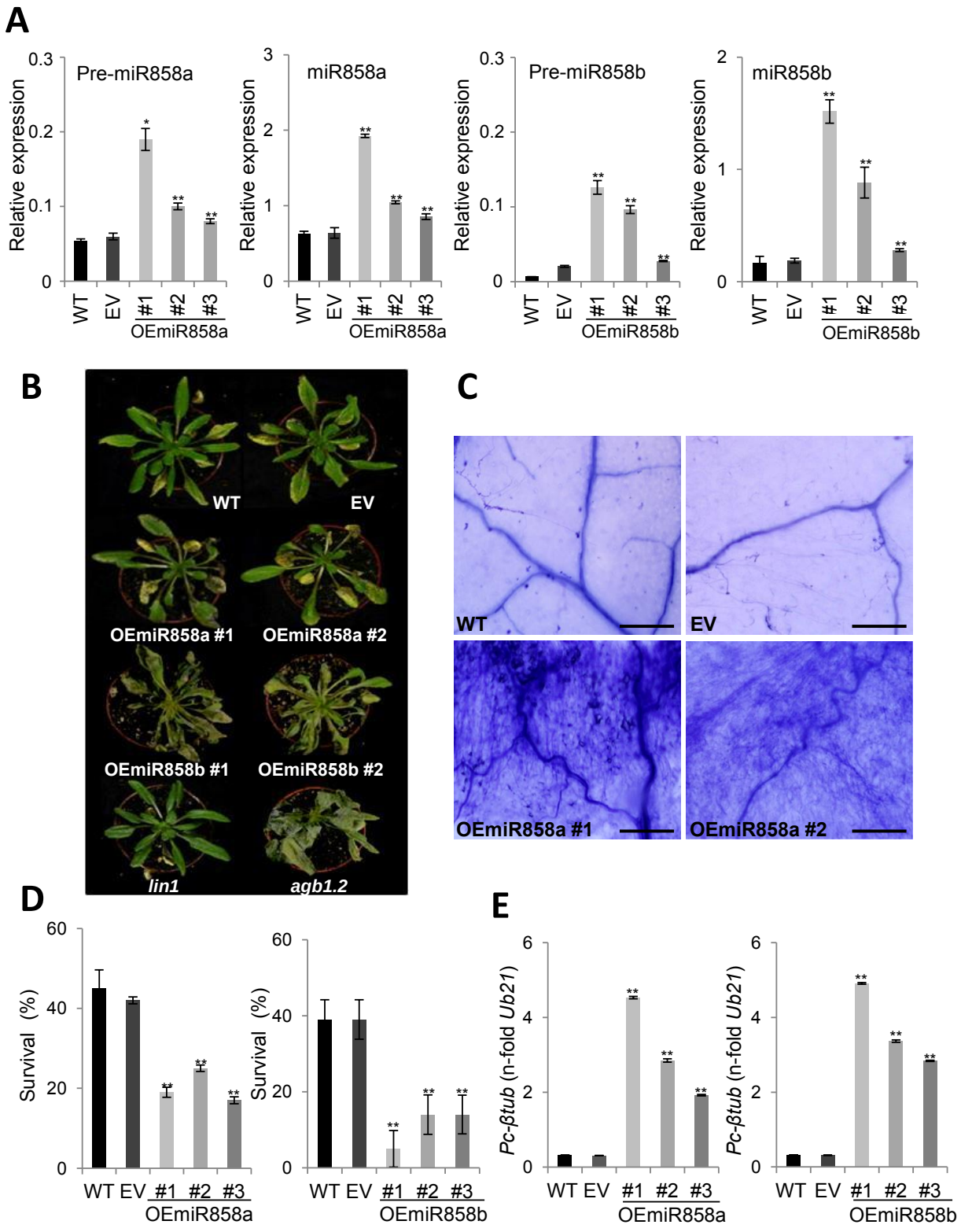


Figure 1. Increased susceptibility to infection by *P. cucumerina* in Arabidopsis plants overexpressing miR858a or miR858b. Plants were grown for 3 weeks under a 12h light/12h dark photoperiod condition. **(A)** Accumulation of pre-miR858a and mature miR858a sequences in plants overexpressin miR858 (OE miR858) was determined by RT-qPCR and stem-loop PCR, respectively. Statistical significance was determined by ANOVA (*, P ≤ 0.05; **, P ≤ 0.01), overexpressor *vs* wild-type or empty vector). **(B)** Phenotype of miR858 overexpressor plants (OE miR858a, miR858b) at 7 days after inoculation with *P. cucumerina* spores ($1x10^6$spores/ml). *agb1.2* and *lin1* plants were used as controls (enhanced susceptibility and resistance to this pathogen, respectively). Results from one of three independent experiments are shown, each with three independent homozygous transgenic plants for each genotype (OE miR858a, OE miR858, empty vector, *lin1* and *agb1.2* plants; at least 24 plants per genotype). **(C)** Trypan blue staining of *P. cucumerina*-infected leaves of OE miR858a plants at 72 hpi. Similar results were obtained for OE miR858b plants. Bars represent 200 µm. **(D)** Survival of OE miR858a and OE miR858b plants was determined at 15 dpi. **(E)** Fungal biomass in OE miR858a and OE miR858b plants at 3 dpi (fungal DNA levels relative to the Arabidopsis *Ubiquitin21* gene).

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

The use of transgenic plants designed to interfere with the activity of specific miRNAs through the target mimicry strategy (*MIM* plants) has proven to be a valuable resource to investigate the function of plant miRNAs, including those involved in immunity (Todesco et al., 2010; Soto-Súarez et al., 2017). Target mimicry is an endogenous regulatory mechanism that plants use to negatively regulate the activity of specific miRNAs (Franco-Zorrilla et al., 2007). Here, 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, which abolishes the cleavage effect (Franco-Zorrilla et al., 2007). In this way, *IPS1* serves as a decoy for miR399 and interferes with the binding of miR399 to its target, thus, preventing miR399 function, and target this miRNA for degradation.

In this work, Arabidopsis plants expressing an artificial target mimic designed to knockdown miR858 (named as *MIM858* plants) were examined for pathogen resistance. The production of *MIM858* plants was previously described (Todesco et al., 2010). Although to a different extent, the accumulation of miR858a and miR858b sequences was reduced in *MIM858* plants compared to control wild-type and transgenic empty vector plants, indicating effective knocking down of the two miR858 family members in these plants (Fig. 2A). Most importantly, *MIM858* plants exhibited enhanced resistance to *P. cucumerina* infection (Fig. 2B; Supplemental Fig. 2). Depending on the line, 56-88% of the *MIM858* plants survived at 15 dpi, but only 21% of the wild-type and 28% of the empty vector plants survived (Fig. 2C). 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. 2D). qPCR analysis also revealed reduced *fungal biomass* in *MIM858* plants compared to control plants (Fig. 2E). The resistance phenotype of *MIM858* plants to *P. cucumerina* infection is consistent with the phenotype of susceptibility observed in OE miR858 plants.

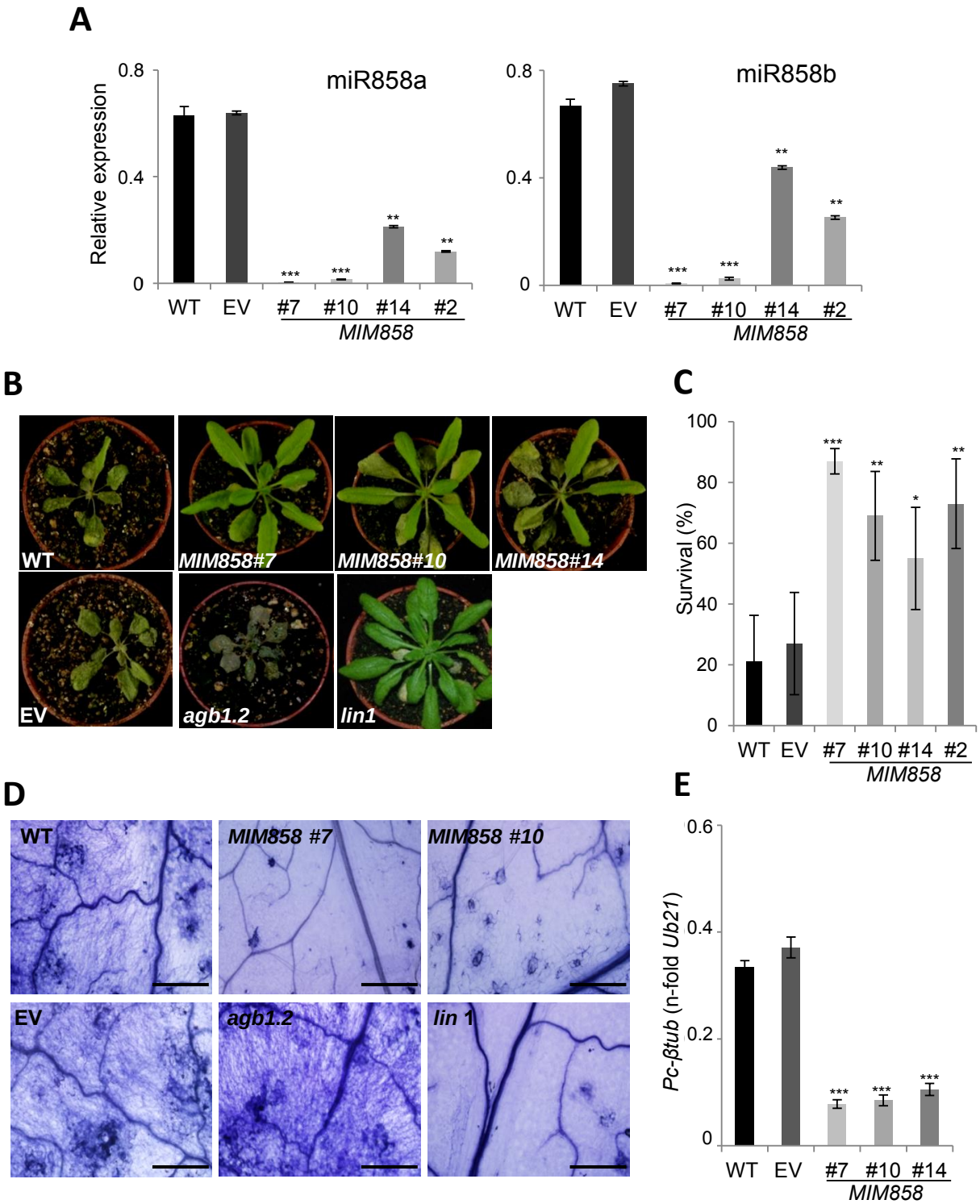


Figure 2. Resistance of *MIM858* plants to infection by the necrotrophic fungus *P. cucumerina*. Plants were grown for 3 weeks under a 12h light/12h dark photoperiod. Four independent *MIM858* lines

were examined. The statistical significance was determined by ANOVA, (*, P ≤ 0.05, **, P ≤0.01, ***, P ≤ 0.001). Histograms show the mean ± SD. **(A)** Accumulation of miR858a and miR858b in *MIM858* plants, as determined by stem-loop RT-qPCR. **(B)** Appearance of wild-type (WT, Col-0), empty vector (EV) and *MIM858* plants at 7 days after inoculation with fungal spores ($4x10^6$spores/ml). Results are from one out of three independent experiments (4 independent *MIM858 lines*, 24 plants/genotype), which gave similar results. **(C)** Survival rates of *MIM858* and control plants at 15 dpi. Results are from three biological replicates for each genotype (24 plants, each replicate). **(D)** Trypan blue staining of *P. cucumerina*-infected leaves at 72hpi. Bars represent 500µm. **(E)** Relative quantification of *P. cucumerina* DNA in wild-type, empty vector and *MIM858* plants at 3 days post-inoculation using specific primers of *P. cucumerina β-tubulin* (Sanchez-Vallet et al., 2012). Values are fungal DNA levels normalized against the Arabidopsis *Ubiquitin21* gene (*At5g25760*). Results are from one out of three independent experiments (24 plants/genotype), which gave similar results.

Next, we investigated whether interference with miR858 activity confers resistance to fungal pathogens with a hemibiotrophic lifestyle. The fungal pathoges here assayed were: *Fusarium oxysporum* f. sp. *conglutinans* (*FOC*), the causal agent of the wilt disease on a broad range of plant species, including *A. thaliana* (Mauch-Mani and Slusarenko, 1994), and *Colletotichum higginsianum* which causes anthracnose leaf spot disease on many cultivated forms of *Brassica* species, and also infects *A. thaliana* (O'Connel et al., 2004, 2012). Upon FOC inoculation, chlorosis and leaf curling was evident in control plants (wild-type, empty vector), culminating in yellowing and necrosis, whereas *MIM858* lines exhibited much milder symptoms (Figure 3A, upper panel). The *MIM858* plants also displayed enhanced resistance to infection by *C. higginsianum* (Figure 3B, upper panel). In agreement with visual inspection of the fungal-infected plants, *MIR858* plants that have been infected with either *FOC* or *C. higginsianum* exhibited higher survival rates, reduced percentage of diseased leaf area and less fungal biomass relative to control plants (wild-type and empty vector) (Figure 3A, B, lower panels). The *MIM858* plants showed normal phenotype as judged by estimation of rosette leaf number and diameter (Supplemental Figure 3).

Collectively, results obtained in disease resistance assays in OE miR858 and *MIM858* plants indicated that *MIR858* plays a crucial role in disease resistance in Arabidopsis plants. Whereas overexpression of miR858 increases susceptibility to infection by fungal pathogens, interference with miR858 activity results in enhanced resistance to pathogen infection (Figs. 2 and 3, respectively). Accordingly, the mechanism underlying pathogen resistance in *MIM858* plants was further investigated.

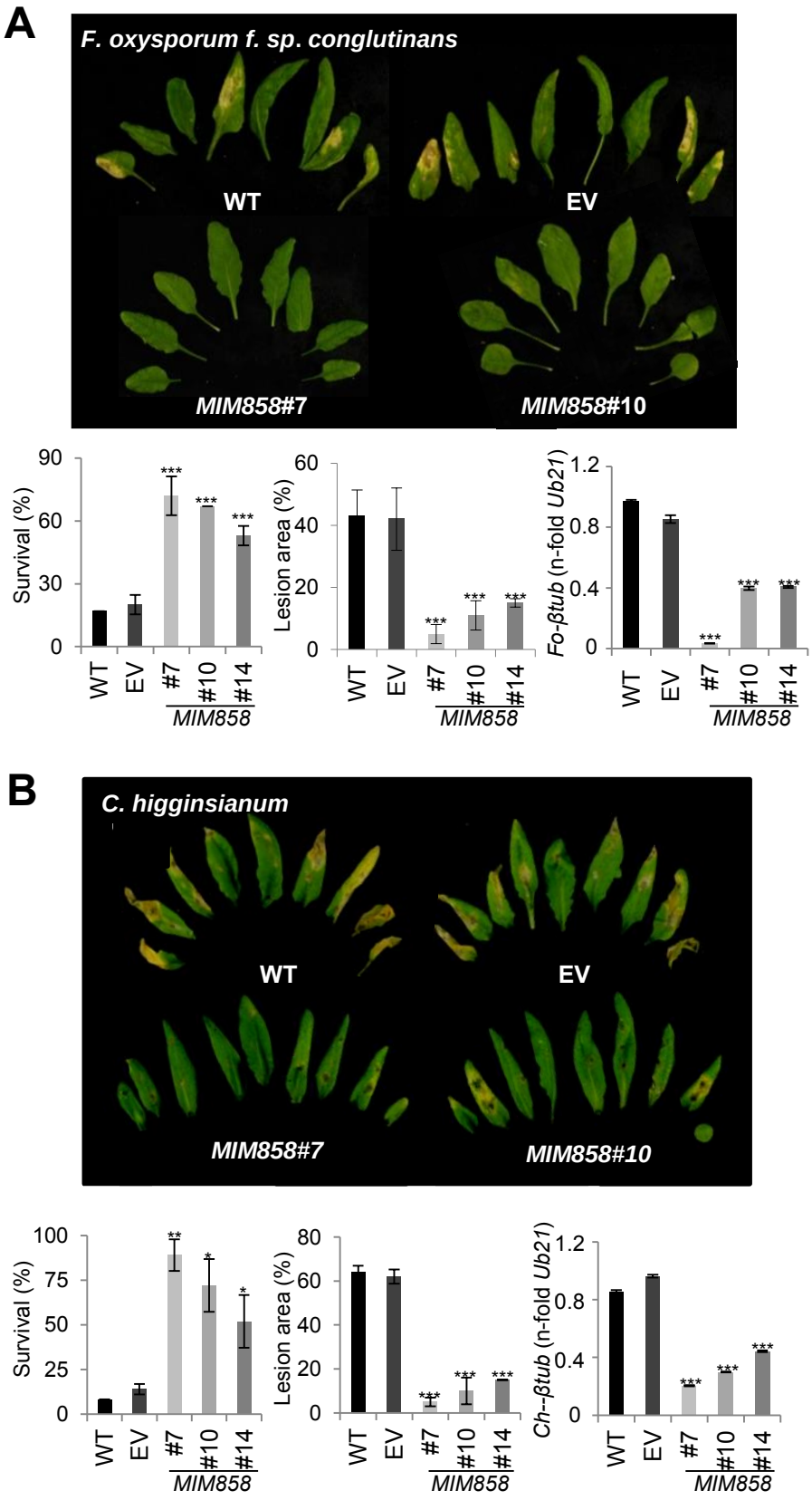


Figure 3. Resistance of *MIM858* plants to infection by the fungal pathogens *F. oxysporum* f.sp. *conglutinans* (*FOC*) (A) and *C. higginsianum* (B). Plants were grown for three weeks under 12h light/12 dark conditions and then inoculated with fungal spores or mock-inoculated. Three independent *MIM858* lines were assayed. As controls, wild-type (WT), and empty vector (EV) plants were used. Histograms show the mean ± SD of three biological replicates, each with at least 24 plants per genotype (ANOVA test,

*, P ≤ 0.05; **, P ≤ 0.01 determined by ANOVA). (**A)** Rosette leaves *MIM858*, wild-type and empty vector plants at 15 days after inoculation with FOC. The inoculum was added to the soil near the base of the plant (200µl/plant; $1x10^6$spores/ml). Leaf area with disease symptoms and survival rate was recorded at 15 dpi and 30dpi, respectively (lower panels). Quantification of fungal DNA in leaves of *FOC*-inoculated plants was carried out by qPCR at 10 dpi relative to the Arabidopsis *Ubiquitin21* gene (lower panels). (**B)** Rosette leaves of *MIM858*, wild-type and empty vector plants at 7 dpi after inoculation with *C. higginsianum* ($1x10^6$ spores/ml). Diseased leaf area and survival was quantified 7 dpi and 15 dpi, respectively (right panels). Fungal DNA levels were determined 3 dpi relative to the Arabidopsis *Ubiquitin21* gene (right panel).

Expression of defense-related genes in *MIM858* plants

The expression of defense-related genes that are known to be regulated by the JA, ET and SA signaling pathways was examined in non-infected and *P. cucumerina*-infected wild type and *MIM858* plants. The genes under study were: *PDF1.2* and *PR4* (markers of JA and ET signaling), *VSP2* and *LOX2* (markers of JA signaling), and *PR1a* and *NPR1* (markers of SA signaling). Whereas the ET and JA pathways are required for *PDF1.2* induction (Penninckx et al., 1998), *PR4* (also known as *HEL*, *hevein-like*) is used as a marker for ET signaling (Lawton et al., 1994). As expected, the expression of these defense genes was induced in wild type plants in response to fungal infection (Figure 4A-C). To note, *PDF1.2* and *PR4* expression (JA/ET marker genes) was induced at a much higher level in the fungal-infected *MIM858* plants compared to the fungal-infected wild-type plants (Figure 4A). Regarding *PDF1.2*, there are many examples in the literature of resistance to pathogen infection in Arabidopsis with enhanced *PDF1.2* expression, including resistance to *P. cucumerina* (Coego et al., 2005; Berrocal-Lobo et al., 2002). When examining the expression of the JA markers (*VSP2*, *LOX2*), or the SA markers (*PR1a*, *NPR1*), their expression was induced at a lower level in *MIM858* plants compared to wild type plants (Figure 4B-C).

Knowing that during pathogen infection there is a super-induction of ET-regulated genes in *MIM858* plants, it was of interest to examine the expression of key genes in ethylene biosynthesis. As it is shown in Figure 4D, *ACC synthase* (*ACS, aminocyclopropane-1-carboxylic acid synthase*) and *ACO* (*ACC oxidase*) expression was induced at a higher level in *MIM858* plants compared to wild-type plants, which is in good correlation with the strong induction of *PDF1.2* and *PR4* expression in these plants upon pathogen challenge.

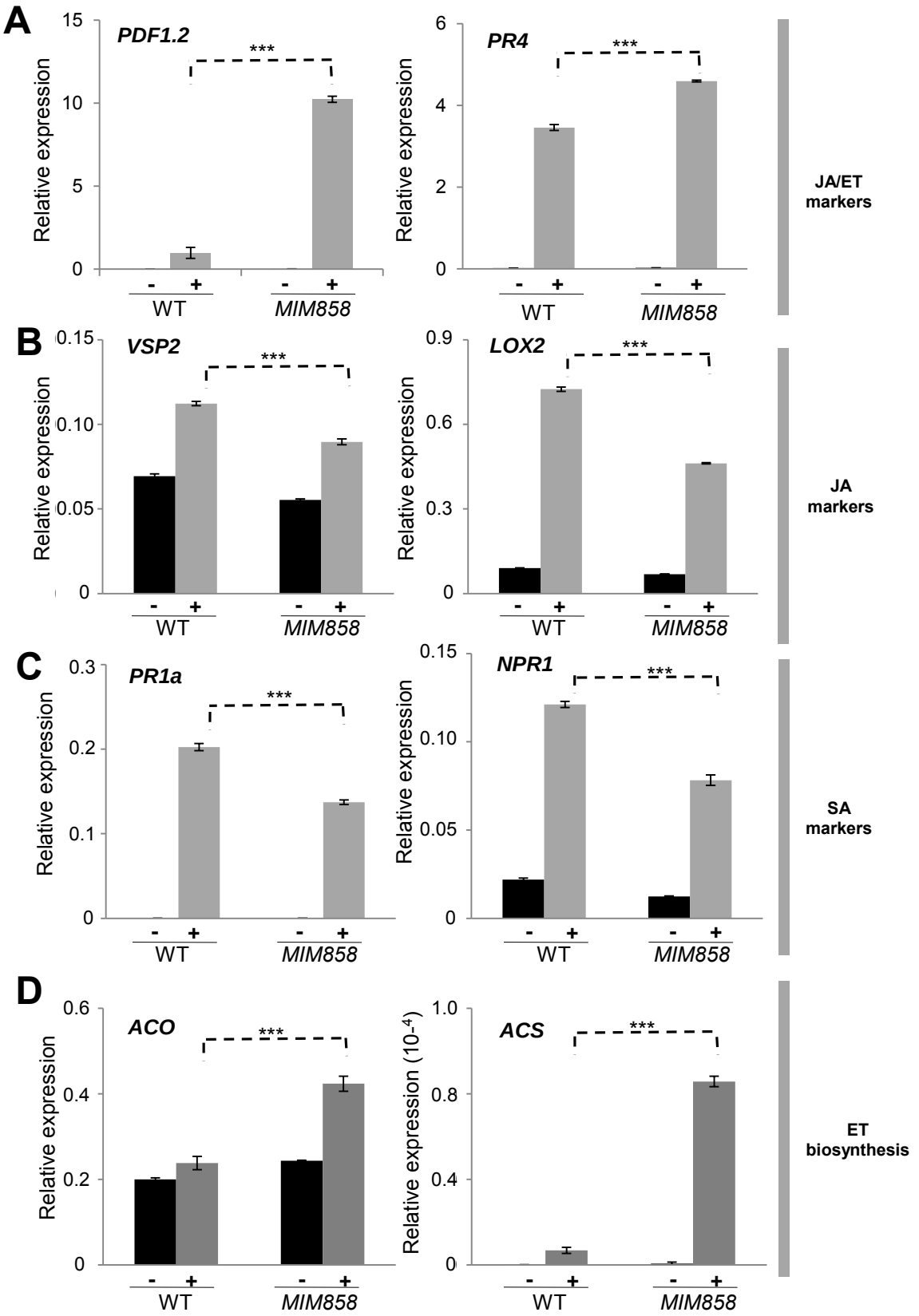


Figure 4. Expression of defense related genes in *MIM858* plants and wild-type plants in response to *P. cucumerina* infection. Three-week old wild type and *MIM858* plants were inoculated with *P. cucumerina* spores ($4x10^6$ spores/ml). Expression analyses were carried out by RT-qPCR at 3 days after

inoculation. Mock-inoculated and *P. cucumerina*-inoculated plants are represented by black and grey bars, respectively. Histograms show the mean ± SD of 3 biological replicates, each with 24 plants per genotype. Statistical significance was determined by ANOVA (***P ≤ 0.001). **(A)** Expression of genes associated with JA and ET-dependent (*PDF1.2*, *PR4*) defense responses. **(B)** Expression of genes associated with JA-dependent (*VSP2*, *LOX*) defense responses. **(C)** Expression of genes associated with SA-dependent (*PR1*, *NPR1*) responses. **(D)** Expression of genes involved in ET biosynthesis (*ACS*, *ACO*).

From these results, it is concluded that interference with miR858 activity in *MIM858* plants results in stronger induction of ethylene biosynthesis (*ACO*, *ACS*), and ET-regulated defense genes (*PDF1.2, PR4*). *MIR858* appears to function as a negative regulator of the ET-dependent signaling pathways in the Arabidopsis response to *P. cucumerina* infection. As for SA-regulated genes, blocking miR858 activity represses the induction of these genes during pathogen infection.

Down-regulation of *MIR858* expression in response to fungal infection and treatment with fungal elicitors.

To gather further support for the involvement of miR858 in Arabidopsis immunity, we examined *MIR858* expression during infection of wild type plants with *P. cucumerina*. A clear reduction in the accumulation of miR858a and miR858b precursors (pre-miR858a and pre-miR858b) could be observed during the entire period of infection here examined (24, 48 and 72 hours post-inoculation), which correlated well with a decrease in the accumulation of the corresponding miR858 mature sequences (Figure 5A). A similar trend in miR858 accumulation (precursor and mature sequences) occurred in Arabidopsis plants that have been treated with a crude preparation of elicitors obtained by autoclaving and sonicating *P. cucumerina* mycelium. That is, miR858a and miR*858b* accumulation decreased in elicitor-treated plants compared to mock-treated plants (Figure 5B).

Next, we investigated whether the reduced level of pre-miR858 transcripts in fungal-infected plants was the consequence of a reduced activity of the *MIR858* promoter. For this, transgenic plants bearing the *β-glucuronidase* (*GUS*) reporter gene under the control of the *MIR858A* promoter (*promMIR858a::GUS*) were generated. Homozygous *promMIR858a::GUS* plants were inoculated with *P. cucumerina* spores or mock inoculated and subjected to histochemical analysis of GUS activity. 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 (non-infection conditions) (Figure 5C, *promMIR858a::GUS*). However, a remarkable decrease in

MIR858a promoter activity occurred in the *P. cucumerina*-inoculated *promMIR858::GUS* plants that was not observed in the control *prom35SCaMV::GUS* plants (Figure 5C). These results confirmed the transcriptional repression of *MIR858A* expression during *P. cucumerina* infection. Not only pathogen infection, but also treatment with fungal elicitors results in down-regulation of *MIR858* expression, suggesting that miR858 play a role in PTI.

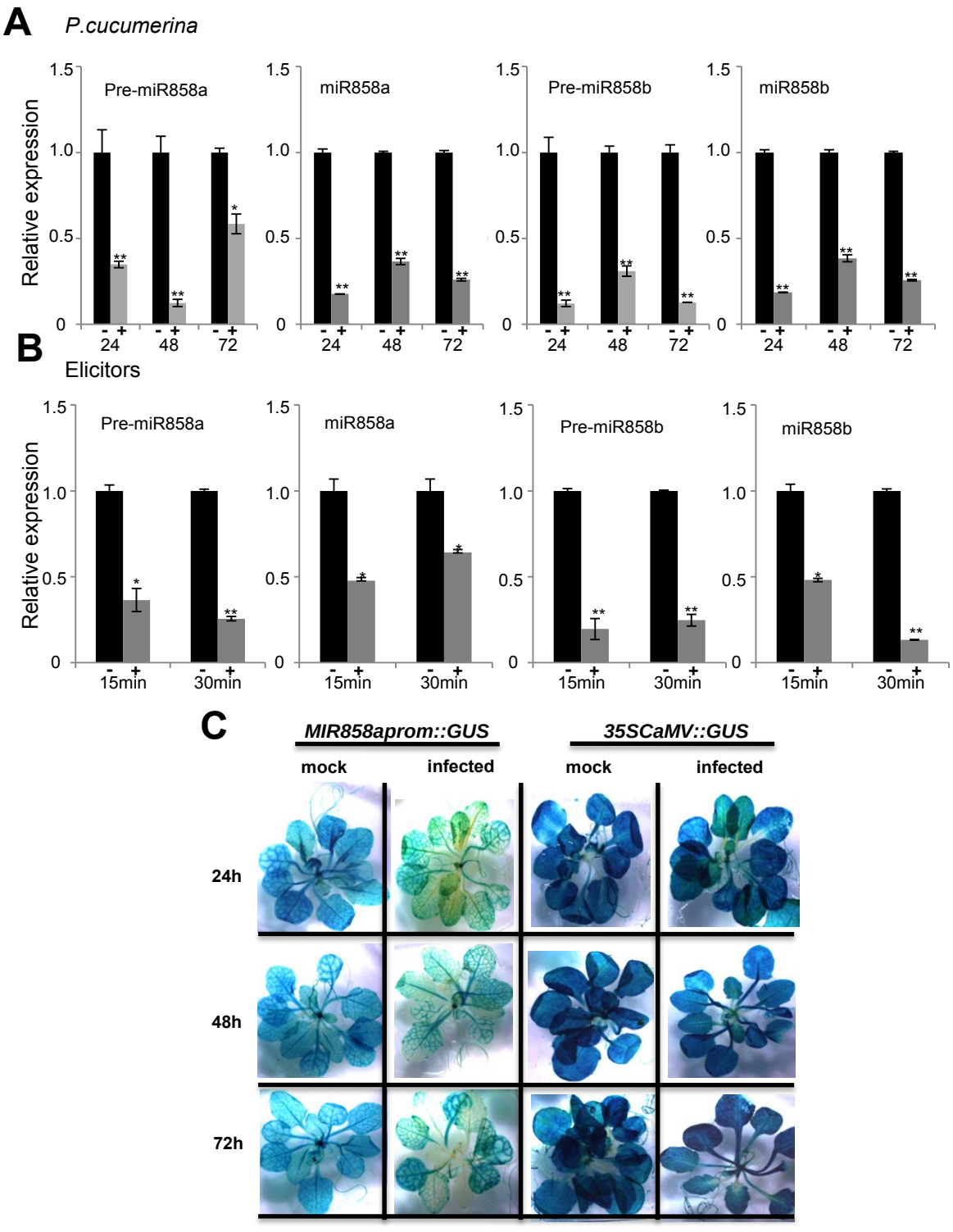


Figure 5. Transcriptional regulation of MIR858 expression during pathogen infection. (A) Accumulation of pre-miR858 and mature miR858 sequences was determined by RT-qPCR analysis and stem-loop RT-qPCR, respectively in wild type plants. Mock-inoculated and *P. cucumerina*-inoculated plants are indicated by black and grey bars, respectively at the indicated times after inoculation. The expression level in mock-inoculated plants was set to 1.0. Statistical significance was determined by ANOVA (*, $P \leq 0.05$; **, $P \leq 0.01$). **(B)** Accumulation of pre-miR858 and mature miR858 sequences in wild-type plants that have been treated with elicitors obtained from the fungus *P. cucumerina*, or mock-inoculated, at the indicated times of treatment. **(C)** Histochemical analysis of GUS activity in *MIR858aprom::GUS* plants that have been mock-inoculated or inoculated with *P. cucumerina* spores (24, 48 and 72 hours post-inoculation). As control, *prom35SCaMV::GUS* plants were used.

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

As previously mentioned, miR858 controls the expression of distinct members of the large family of *MYB* transcription factors. A miR858-guided cleavage of *AtMYB11*, *AtMYB12*, *AtMYB13*, *AtMYB20* and *AtMYB111* is documented (Fahlgren et al., 2007; Addo-Quaye et al., 2008; Sharma et al., 2016). Several other *MYB* genes are predicted as target genes for miR858, but their validation as miR858 targets is still lacking.

In general, if a miRNA is down-regulated, its target genes are likely to be up-regulated. Consistent with this notion, down-regulation of miR858 in *MIM858* plants results in up-regulation of miR858 target genes (e.g. *AtMYB11*, *AtMYB12, AtMYB13, AtMYB20* and *AtMYB111*) (Figure 6A). This finding indicates that the inactivation of miR858 activity by target mimics is functional and results in increased expression of flavonoid-specific *MYB* transcription factor genes (*AtMYB11*, *AtMYB12*, *AtMYB111*). *AtMYB11*, *AtMYB12* and *AtMYB111* expression was also found to be up-regulated by fungal infection in wild type plants (Supplemental Figure 4), which is consistent with the reduction that is observed in these plants during infection (see Figure 5A).

The Arabidopsis *AtMYB11, AtMYB12* and *AtMYB111* transcription factors function as activators of flavonoid synthesis through activation of *Chalcone synthase* (*CHS*)*, Chalcone Isomerase* (*CHI*)*, Flavonol-3-hydroxylase* (*F3H*) and *Flavonol Synthase1* (*FLS1*) expression, these genes encoding the enzymes that enzymes catalyze the production of flavonols from 4-coumaroyl-CoA (Figure 6B) (Mehrtens et al., 2005; Stracke et al., 2007; Liu et al., 2015). An advantage of using Arabidopsis for studying flavonoid biosynthesis is that single copy genes encode all enzymes of the central flavonoid metabolism, with the exception of flavonol synthase (*FLS*), which is encoded by six genes, but only two (*FLS1* and *FLS3*) have demonstrated activity (Owens et al., 2008; Preuss et al., 2009). Recently, Sharma et al (2016) reported that *CHS*, *CHI*, *F3'H* and *FLS1* expression is up-regulated in *MIM858* plants, which is consistent with the observed

up-regulation of *AtMYB11*, *AtMYB12* and *AtMYB111* expression in these plants. A metabolomic analysis of *MIM858* plants revealed flavonoid accumulation in these plants (Sharma et al., 2016).

We speculated that miR858-mediated alterations in *AtMYB11*, *AtMYB12* and *AtMYB111* expression in *MIM858* plants could have an effect on the expression of phenylpropanoid genes beyond its effect on the target genes for the miR858-regulated flavonoid-specific *MYB* transcription factors (e.g. *CHS*, *CHI*, *F3H*, *FLS*). To test this possibility, we examined the expression of genes acting upstream of the flavonoid branch in the general phenylpropanoid pathway, namely the genes involved in the production of *p-coumaril-CoA* from phenylalanine (Figure 6B). They genes were: *PAL* (*phenylalanine ammonia-lyase*), *C4H* (*cinnamate-4-hydroxilase*) and *4CL* (*4-Coumarate-CoA-ligase*). Regarding *C4H* and *4CL*, these genes were found to be up-regulated in *MIM858* plants compared to wild type plants (Figure 6C). In agreement with this, *C4H* and *4CL* expression was down-regulated in OE miR858 plants (Supplemental Figure S5). Regarding *PAL* expression, in Arabidopsis there are 4 *PAL* genes which differentially respond to different types of stress (Huang et al., 2010). Of them, only *PAL4* expression was found to be up-regulated in *MIM858* plants. As for the other *PAL* genes, *PAL1* and *PAL 3* expression decreased in *MIM858* plants, whereas *PAL2* expression was not affected in these plants (Supplemental Figure 6). From these results it is concluded that the functional blockage of miR858 activity has consequences that go beyond alterations in flavonoid-specific *MYB* genes (*AtMYB11, AtMYB12, AtMYB111*), and that genes upstream of the flavonoid branch of the phenylpropanoid pathway (*PAL4*, *C4H* and *4CL*) are also up-regulated in *MIM858* plants.

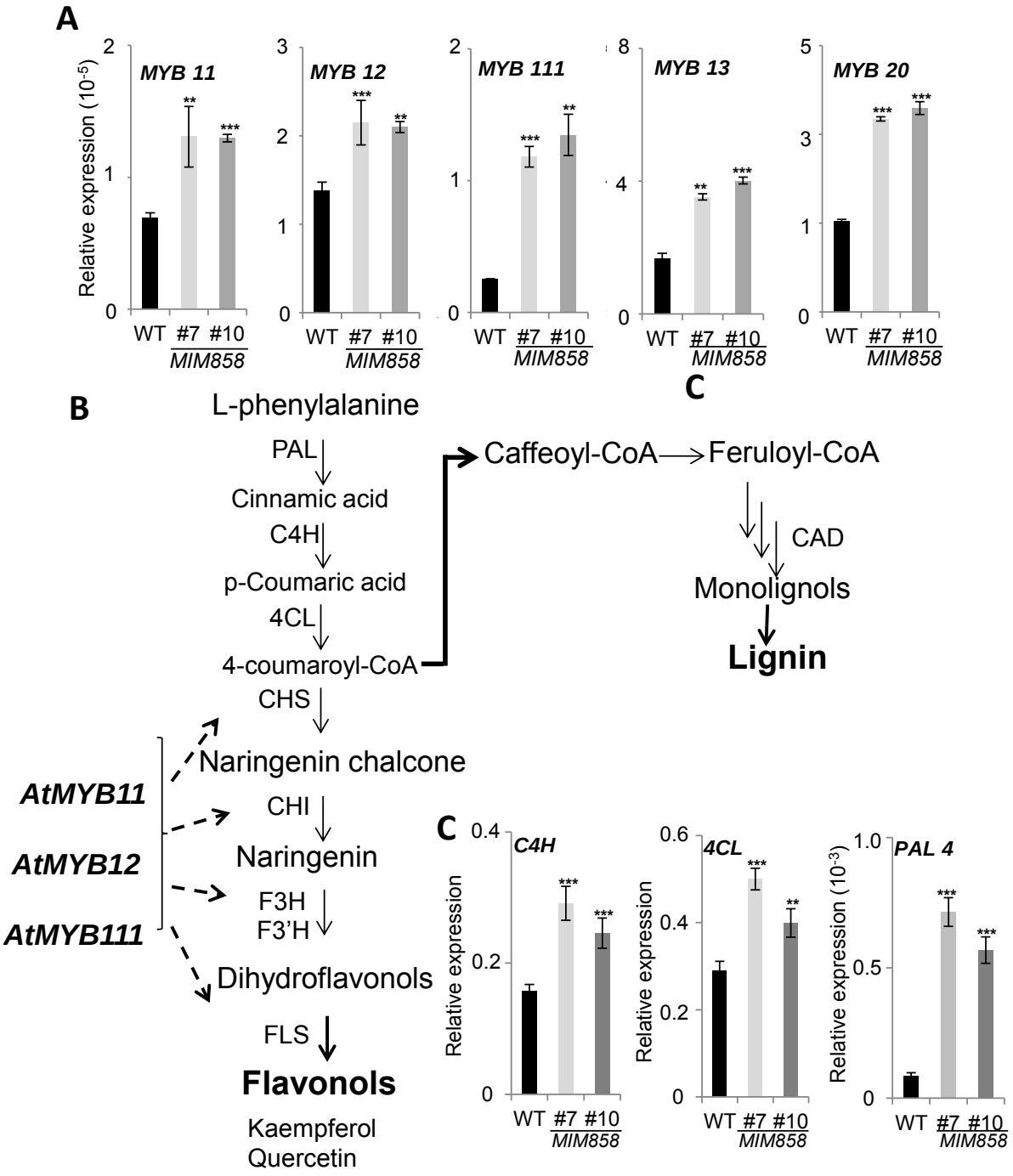


Figure 6. Expression of genes in the general phenylpropanoid pathway. RT-qPCR analysis was carried out using the *β-tubulin2* gene (*At5g62690*) as the internal control. Statistical significance was determined by ANOVA (*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001). Histograms show the mean ± SD of 3 biological replicates, each with 24 plants per genotype. **(A)** Expression of miR858 target genes (*AtMYB11*, *AtMYB12*, *AtMYB111, AtMYB13* and *AtMYB20*. **(B)** Scheme of branch pathways of the phenylpropanoid pathway leading to flavonols and lignin. miR858 regulates the expression of *MYB* transcription factors which function as activators of the early flavonoid biosynthetic genes in the phenylpropanoid pathway (*AtMYB11*, *AtMYB12*, *AtMYB111*). Abreviations are as follows: *CAD*, *cinnamyl-alcohol dehydrogenase*; *C4H*, *Cinnamate-4-hydroxylase*; *4CL*, *4-Coumarate-CoA Ligase*; *CHI*, *Chalcone isomerase*; *CHS*, *Chalcone synthase*; *F3H*, *Flavonol-3-hydroxylase*; *F3'H*, *Flavonol-3'-hydroxylase*; *FLS*, *Flavonol synthase*; *PAL*, *Phenylalanine ammonia-lyase*. **(C)** Expression of genes involved in the early steps of the general phenylpropanoid biosynthesis pathway (*C4H*, *4CL* and *PAL4*) leading to the production of *4-coumaroyl-CoA*, the precursor for the flavonoid and lignin branches of the phenylpropanoid pathway.

Knowing that *MIM858* plants exhibited enhanced resistance to fungal infection, we sought to investigate whether flavonoids are relevant in conferring the phenotype of disease resistance that is observed in these plants. Flavonoid accumulation was examined in leaves of mock-inoculated and *P. cucumerina*-inoculated wild-type, *MIM858* and OE miR858 plants. DPBA (diphenylboric acid 2-aminoethyl ester) staining was used to visualize flavonoid accumulation (Buer et al., 2007; Lewis et al., 2011). DPBA binds to flavonoids and fluoresces *in vivo,* and the flavonoid-DPBA conjugates have unique fluorescent color (e.g. yellow-green fluorescence correspond to DPBA bound to the flavonol kaempferol, fluorescing at 520 nm) (Peer et al., 2001; Saslowsky and Winkel-Shirley, 2001). Microscopic analysis of DPBA-stained leaves revealed clear differences in flavonoid accumulation between *MIM858* and WT plants. Whereas only a few tiny green-fluorescence signals were distinguishable on the leaf surface of wild type plants, larger regions showing intense green-yellow fluorescence were consistently found in leaves of *MIM858* plants (mock-inoculated plants in both cases) (Figure 7A). The green-yellow fluorescence of flavonoid-DPBA conjugates that is observed in *MIM858* plants might well correspond to kaempferol-DPBA conjugates, as previously reported by 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). Most importantly, we noticed that whereas the fluorescence localized to discrete regions in leaves of mock-inoculated plants (both wild-type and *MIM858* plants), the flavonoid-DPBA fluorescence exhibited a more generalized distribution in *P. cucumerina*-infected leaves of both wild-type and *MIM858* plants (Figure 7A). For a comparison, flavonoid-DPBA fluorescence accumulation was not detected in OE miR858 plants (Figure 7A).

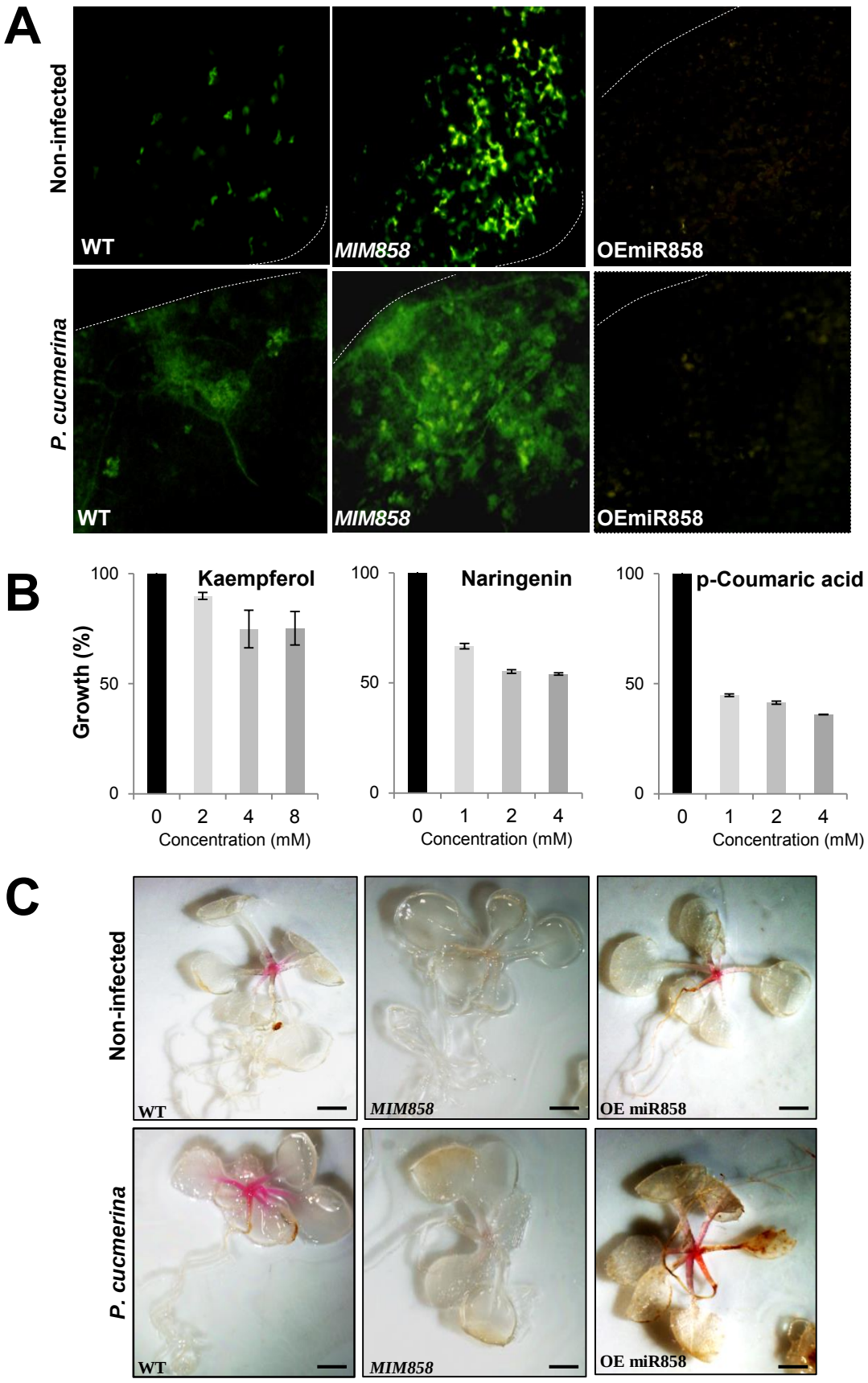


Figure 7. ***In situ*** **flavonoid detection, antifungal activity of phenylpropanoid compounds, and lignin accumulation in wild-type,** ***MIM858*** **and OE miR858 plants. (A)** Flavonoid accumulation was visualized

by DPBA staining of rosette leaves. Two week-old plants were inoculated with *P. cucumerina* spores ($1x10^5$spores/ml), or mock-inoculated, and subjected to DPBA staining at 72 hpi. DPBA-flavonoid conjugate fluorescence was recorded in the green channel (λ_{exc} = 488 nm; acquisition in the 560–600-nm wagelength). **(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 48h in PDB medium in the presence of increasing concentrations of the corresponding metabolite. Fungal growth is expressed as 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. **(C)** Lignin accumulation in wild-type, *MIM858* and OE miR858a plants was determined by whole-mount phloroglucinol staining (B). Pictures were taken at 72 hpi with an Olympus DP71 camera. Bars represent 1 mm.

Next, we hypothesized that the increased levels of flavonoids accumulating in *MIM858* plants might be responsible of the phenotype of resistance that is observed in these plants. For this reason, in this work we investigated the possible antifungal activity of the flavonoids naringenin and kaempferol, as well as the phenylpropanoid p-coumaric acid. *In vitro* antifungal activity assays revealed that the two flavonoids here assayed, kaempferol and naringenin inhibited *P. cucumerina* growth, naringenin having a greater antifungal activity than kaempferol (Figure 7B). When increasing the concentration of naringenin above 2 mM, or kaempherol above 4 mM, no further increase in antifungal activity could be observed indicating that the potency of these compounds against *P. cucumerina* might have reached maximum values under the experimental conditions here assayed. The phenylpropanoid p-Coumaric acid was found to be more effective for inhibition of *P. cucumerina* growth than each one flavonoid (naringenin, kaempferol) (Figure 7B). After 48 of incubation, a concentration of 1mM of p-Coumaric acid results in 55% inhibition of *P. cucumerina* growth (45% growth of control cultures). As it was observed with the two flavonoid compounds, increasing the concentration of p-Coumaric acid did not significantly increase the antifungal potency of this compound. Microscopical observations of fungal cultures revealed alterations in the morphology of hyphae in cultures that have been grown in the presence of one or another compound. Hyphae with constricted regions were frequently observed in treated-cultures compared the control cultures (Supplemental Figure 7). It is then reasonable to assume that the accumulation of flavonoids (e.g. kaempferol, naringenin) and phenylpropanoid (e.g. p-Coumaric acid) compounds exhibiting antifungal activity in *MIM858* plants might be responsible, at least in part, of the disease resistance phenotype in these plants.

Pathogen resistance in *MIM858* plants does not requires lignification

Lignin monomers are produced from p-coumaroyl CoA, the branch point for the production of flavonoids and monolignols in the phenylpropanoid biosynthetic pathway (see Figure 6B). It is generally assumed that lignin plays a role in resistance to pathogen infection in different pathosystems (Miedes et al., 2014). Lignin is deposited in the secondary cell wall, thus, providing a physical barrier against pathogen invasion. Along with this, a positive correlation between lignin accumulation and resistance toward pathogens has been observed in Arabidopsis plants (Miedes et al., 2014). However, the observation that *MIM858* plants had reduced lignification in the vascular and interfascicular tissues (Sharma et al., 2016) argued against the idea that lignin accumulation might protect the *MIM858* plants from pathogen infection. Accordingly, in this work we investigated whether resistance to *P. cucumerina* infection in *MIM858* plants is accompanied by lignification.

Lignin accumulation in mock-inoculated and *P. cucumerina*-inoculated wild-type, *MIM858* and OE miR858 plants was examined using the whole-mount phloroglucinol staining method. In the absence of pathogen infection, lignin was detected in wild-type as well as in OE miR858 plants, but not in *MIM858* plants (Figure 7C, upper panels). Consistent with the observed reduction in lignin content, the expression of the lignin-specific *CAD* (*cinnamyl alcohol dehydrogenase*) genes (*CAD5*, *CAD6*) was down-regulated in *MIM858* plants compared to wild-type plants (Supplemental Figure 8). *CAD* enzymes synthesize cinnamyl alcohols that are the immediate precursors of lignin.

When examining *P. cucumerina* infected plants, lignin accumulation increased in wild-type plants and OE miR858 plants, but not in *MIM858* plants (Figure 7C, upper panels), supporting that inhibition of miR858 activity by the target mimics strategy is effective for inhibition of lignin biosynthesis during fungal infection. From these results, it is concluded that lignification does not contributes to pathogen resistance in *MIM858* plants. Instead, down-regulation of miR858, and concomitant up-regulation of miR858 target genes involved in flavonoid biosynthesis, might contribute to pathogen resistance in *MIM858* plants by re-directing the metabolic flux towards the production of antifungal phenylpropanoid compounds (including flavonoids), away from lignin biosynthesis.

Discussion

In this study we provide evidence that miR858 is involved in Arabidopsis immuninty. Firstly, we demonstrated that overexpression of miR858 renders Arabidopsis plants more susceptible to infection by the necrotrophic fungal pathogen *P. cucumerina*. Conversely, inhibition of miR858 activity by target mimics results in enhanced resistance to fungal pathogens, both necrotrophic (*P. cucumerina*) and hemibiotrophic (*F.oxysporum* f. sp. *conglutinans*, *C. higginsianum*) pathogens, further supporting that miR858 is a negative regulator in Arabidopsis immunity. Secondly, *MIR858* expression was found to be transcriptionally down-regulated during *P. cucumerina* infection in wild type with *P. cucumerina*. Not only pathogen infection, but also treatment with fungal elicitors is accompanied by down-regulation of *MIR858*, indicating that miR858 is a component of PTI. The increased resistance to fungal infection that is observed in *MIM858* plants is associated to a stronger induction of ET-dependent defense responses (*PDF1.2*, *PR4*) and ET biosynthesis genes (*ACO*, *ACS*) upon pathogen challenge. In line with this, resistance to infection by *P. cucumerina* was reported to be dependent on the ET signaling pathway in Arabidopsis plants (Berrocal-Lobo et al., 2002). Interactions between the JA and the ET signaling as well as between the JA/ET and SA pathways are known to occur. Although the induction of JA- (*VSP2*, *LOX2*) or SA- (*PR1*, *NPR1*)-regulated genes was less pronounced in *MIM858* plants than in WT plants, additional studies are needed to determine the exact mechanisms by which miR858 activity modulates defense hormone signaling.

In the absence of pathogen infection, defense gene expression is not activated in *MIM858* plants. Only upon pathogen challenge, a superinduction of defense responses occurs in *MIM858* plants which is reminiscent of defense priming, an adaptive strategy of plants to overcome pathogen infection that relies on a faster, stronger, and/or more sustained expression of defense responses upon pathogen challenge (Conrath, 2011; Balmer et al 2015; Conrath et al., 2015; Martinez-Medina et al., 2016).

Visualization of flavonoid accumulation in rosette leaves of wild-type and *MIM858* plants by DPBA staining revealed quantitative and qualitative changes in the accumulation of flavonoids during to fungal infection. Compared to wild-type plants, the *MIM858* plants accumulated higher levels of flavonoids which accumulated at restricted sites under non-infection conditions in both wild-type and *MIM858* plants. Upon *P. cucumerina* infection,

however, flavonoids showed a more general distribution through the leaf in both wild-type and *MIM858* plants. Given that flavonoids have been reported to be capable of long-distance movement in Arabidopsis (Buer et al., 2007), our observation of a generalized distribution of flavonoids in *P. cucumerina*-infected leaves raises the possibility that these compounds might act as signaling molecules for the activation of defense responses. In other studies, flavonoids were proposed to function as signal molecules in auxin transport, or during symbiotic nitrogen fixation and mycorrhizal associations (Buer et al., 2010; Falcone-Ferrreira et al., 2012).

We show that naringenin and kampherol inhibit *P. cucumerina* growth, thus, supporting the idea that flavonoid accumulation contributes to antifungal resistance in *MIM858* plants. p-Coumaric acid, an intermediate in the phenylpropanoid pathway, also exhibited antifungal activity against *P. cucumerina*, its antifungal potency being even higher than that of the two flavonoids assayed in this study. That the three compounds accumulate at higher level in *MIM858* plants compared to wild-type plants is supported by metabolomic data (Sharma et al., 2016). The *in vitro* antifungal activity of the individual phenylpropanoid compounds assayed in this work (p-Coumaric acid, naringenin, kaempferol) is, however, weaker than that of known plant antimicrobial peptides, such as lipid transfer proteins or thionins (Molina et al., 1993a, b). Presumably, the resistance to infection in *MIM858* plants would rely on the simultaneous action and combined effect of antifungal activities of flavonoids (at least naringenin and kaempferol) and phenylpropanoid compounds (e.g. p-Coumaric acid) rather than on the activity of individual phenylpropanoid compounds. In favor of this hypothesis, the induction of genes involved in flavonoid biosynthesis and/or flavonoid accumulation has been reported in different pathosystems (Iqbal et al., 2005; Foster-Hartnett et al., 2007). Certain flavonoids isolated from plant tissues exhibited *in vitro* antimicrobial activity against phytopathogens (Dai et al., 1996; Galeotti et al., 2008; Bollina et al., 2010; De Conti Lourenco et al., 2013; Mierziak et al 2014).

Flavonoids have been proposed to function as ROS scavengers and chelators of metals that might generate ROS via de Fenton reaction, potentially acting as antioxidant molecules in protecting the plant cell from oxidative stress induced by pathogen infection (Falcone Ferreyra et al., 2012; Mierziak et al., 2014). However, the relevance of the antioxidative properties for flavonoid in plant immunity is still a topic of debate. Clearly,

there is still much to learn about the mechanisms by which flavonoids might serve either as antimicrobial agents or endogenous signal molecules in plant/pathogen interactions.

Results here presented indicate that the functional blockage of miR858 activity by target mimics results in up-regulation of miR858 target genes functioning in the flavonoid branch of the phenylpropanoid pathway (e.g. *AtMYB11*, *AtMYB12* and *AtMYB111*). We also show that genes involved in the early steps of the phenylpropanoid pathway, upstream of the flavonoid branch are also up-regulated in *MIM858* plants (e.g. *PAL4*, *C4H* and *4CL*). Most probably, *PAL4*, *C4H*, *4CL* and the miR858-regulated flavonoid-specific *MYB* genes are regulated in a coordinated manner in order to prioritize flavonoid production in *MIM858* plants. However, at present it is not possible to determine from our data whether alterations in the expression of these genes was due to a feed-back control by metabolite levels, or to protein-protein interactions of transcription factors and other regulatory proteins forming transcriptional complexes controlling flavonoid biosynthesis.

Sequestration of miR858 by target mimics also results in down-regulation of genes encoding the specific and last step enzymes for production of monolignols (*CAD5*, *CAD6*) which is consistent with the observed reduction in lignin accumulation in *MIM858* and metabolomic data previously reported on *MIM858* plants (Sharma et al., 2016). Most importantly, lignin accumulation was not observed in *P. cucumerina*-infected *MIM858* plants, supporting that resistance to fungal infection in these plants does not require a lignification response. Thus, down-regulation of miR858 activity might well function to re-direct the phenylpropanoid pathway towards the production of antifungal flavonoids at the cost of lignin synthesis which might represent a plant's natural defense response effective for disease resistance. Knowing that resistance in *MIM858* plants is associated to flavonoid accumulation, and that flavonoids have been reported to accumulate in cell walls during pathogen infection (Dui et al., 1996), it is tempting to hypothesize that a reduced lignification in *MIM858* plants may facilitate the incorporation of flavonoids in host cell walls. Contrary to this, lignification has been associated with resistance to pathogen infection in different plant species (Miedes et al., 2014).

Finally, it is worth mentioning that under our experimental conditions, plants overexpressing either miR858 or the miR858 target mimic grew and developed normally in the absence of pathogen infection. Differences between OE miR858 or *MIM858* and wild type plants were, however, reported in previous studies by Sharma et al (2016). These authors also described a photoperiod-dependent regulation of *MIR858*

expression. A possible explanation for the different phenotypes that are observed in overexpressor and target mimic plants (Sharma et al., 2016; present work) might be the photoperiod conditions used to grow Arabidopsis plants. In our work, the plants were always grown under neutral day condition (12h light/12h dark photoperiod), whereas Sharma et al (2016) grew plants under a long day photoperiod (16h light/8h dark photoperiod). The different growth responses that are observed in *MIM858* plants in neutral and long day conditions might reflect specific but currently unknown mechanisms of adjusting miR858 expression to the prevalent photoperiod condition. It is also known that transcript levels for genes involved in flavonoid biosynthesis accumulate at different levels depending on the photoperiod conditions (Baerenfaller et al., 2005). Further studies are, however, needed to establish whether there are links between light regulation of *MIR858* expression and light-dependent accumulation of flavonoids in the context of disease resistance. Connections between miR858-mediated alterations in phenylpropanoid metabolism and induction of defense responses also need to be elucidated.

To conclude, results here presented demonstrated that alterations on *MIR858* expression have important consequences in disease resistance. Our findings also support 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. Very recently, miR858 was reported to mediate tolerance to drought stress in the desert plant *Ammopiptanthus mongolicus* (Gao et al., 2016). Whether miR858 plays a regulatory role in adaptation to biotic and abiotic stress in Arabidopsis remains to be determined. The information provided in this work extends our knowledge on miRNAs involved in plant immunity and lays the foundation for future research to uncover links between phenylpropanoid metabolism and plant immunity. This study can also provide new ways to develop strategies for plant disease resistance.

Materials and Methods

Plant and fungal materials

Arabidopsis thaliana (ecotype Columbia-0) plants were grown under a 12h light/12h dark photoperiod and 60% relative humidity at a temperature of 22 ± 2°C. For *in vitro* assays, seeds were grown for 14 days on MS medium containing 0.8% agar and vitamins. The Arabidopsis mutants used in this work, *agb1.2* (Llorente et al., 2005) and *lin1* (Malamy et al., 2001; Gamir et al., 2014) were all grown as described above.

Fungi were grown at 28ºC on PDA (potato dextrose agar) supplemented with chloramphenicol (34mg/ml). 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 bp and 428 bp DNA fragments for the miR858a and miR858b precursor, respectively). Primers are listed in Supplemental Table 1). Precursor DNA sequences were cloned into the pCAMBIA1300 binary vector under the control of the *CaMV35S* (*Cauliflower Mosaic Virus 35S*) promoter.

To obtain the *MIR858a promoter:GUS* construct, the DNA sequence of the *MIR858a* promoter region was extracted from 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 (Clough and Bent, 1998). Transgenic lines harboring the empty vector (pCAMBIA1300 or pCAMBIA1391z) were also obtained and used as controls. Transgenic plants were selected on Murashige and Skoog (MS) media supplemented with hygromycin (50mg/ml), and homozygous lines were identified.

Fungal infection and elicitor treatment.

Three-week-old *Arabidopsis* plants were spray-inoculated with a spore suspension of *P. cucumerina* at the appropriated concentration. In each case, at least three independent transgenic lines overexpressing either miR858a or miR858b, or expressing the miR858 target mimic, were assayed. 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 *F. oxysporum f. sp. conglutinans* (FOC) were performed by applying the spore suspension to the soil (200 µl, 10^6spores/ml). Inoculations with *C. higginsianum* were carried out by placing 2 drops of the spore suspension on each leaf; (5µl drops, 10^6 spores/ml). 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 three-week old plants with an elicitor extract obtained from *P. cucumerina* (300 µg/ml) as described (Casacuberta et al., 1992). Three independent infection experiments, or treatment with fungal elicitors, were performed (at least 24 plants per genotype in each experiment). Statistically significant differences among genotypes were determined by one-way ANOVA test.

Lesion areas were quantified with the ASSESS v2.0 software on 4 inoculated leaves per plant and 24 plants per each genotype. Quantification of fungal DNA on infected leaves was carried out by real-time PCR using specific primers for the corresponding fungus and the *UBIQUITIN21* (*At5g25760*) gene as an internal control. PCR primers are listed in Supplemental Table 1.

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 4 h, washed with 70% ethanol for 5 min. Leaves were placed in glass slides with glycerol an observed using a Zeiss Axiophot microscope.

Expression analysis RT-qPCR and stem-loop RT-PCR

Total RNA was extracted from rosette leaves using the TRIzol Reagent (Invitrogen). Reverse transcription reactions were performed using DNase-treated total RNA (1 µg) and reverse transcriptase (Applied Biosystems) and oligo-dT_{18} (Sigma, Aldrich).

RT-qPCR (Reverse transcriptase 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 *beta-tubulin2* gene was used as the internal control for normalization. Three independent biological replicates were analyzed. Specific stem-loop primers were used for miR858 amplification by stem-loop RT-qPCR (ST-RT qPCR). Primers are listed in Supplemental Table 1. ANOVA tests were used to evaluate differences in gene expression.

Histochemical 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 1h in ethanol:formaldehyde:acetic acid (80:3.5:5 v/v), stained with lactophenol blue solution for 4 hours, and washed with 70% ethanol (5 minutes). Leaves were placed in glass slides with glycerol an observed using a microscopy Aixophot DP70.

Determination of lignin content

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

In vivo staining of flavonoids

Flavonoids were visualized in vivo by the fluorescence of flavonoid-conjugated DPBA (diphenylboric acid 2-amino-ethylester) to the compounds after excitation at 488 nm as described by Watkins et al., (2016). Leaves were submerged in a solution containing 0.01% (v/v) Triton X-100 and 2.52 mg/ml DPBA for 2.5 hours. Fluorescence was observed following UV excitation of DPBA-stained leaves on an AixoPhotDP70 microscope (450nm-490nm). DPBA fluoresces yellow-green (Emax = 520 nm) when bound to kaempferol.

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) were mixed with 50 µl of *P. cucumerina* spores (10^6 spores/ml). Spores were allowed to germinate for 6h. The

secondary metabolite was then added to the desired final concentration. The microtiter plates were incubated at 25ºC for 48 hours 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 (0.1mg/ml).

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References

Addo-Quaye C., Eshoo T.W., Bartel D.P., Axtell M.J. 2008. Endogenous siRNA and miRNA targets identified by sequencing of the Arabidopsis degradome. Curr. Biol. 18, 758-762.

Baerenfallera K., Massonnetb C., Henniga L., Russenbergera D., Sulpiced R., Walsha S., et al. 2015. A long photoperiod relaxes energy management in Arabidopsis leaf six. Curr. Plant Biol. 2, 34–45.

Baldrich P., San Segundo B. 2016. MicroRNAs in Rice Innate Immunity. Rice. 9:6.

Balmer A., Pastor V., Gamir J., Flors V., Mauch-Mani B. 2015. The 'prime-ome': towards a holistic approach to priming. Trends Plant Sci. 20, 443-452.

Berrocal-Lobo M., Molina A., Solano R. 2002. Constitutive expression of *ETHYLENE-RESPONSE-FACTOR1* in Arabidopsis confers resistance to several necrotrophic fungi. Plant J. 29, 23-32.

Boller T., Felix G. 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Plant Biol. 60, 379–406.

Boller T., He S.Y. 2009. Innate Immunity in Plants: An Arms Race Between Pattern Recognition Receptors in Plants and Effectors in Microbial Pathogens. Sci. 324, 742–744.

Bollina V., Kumaraswamy G.K., Kushalappa A.C., Choo T.M., Dion Y., Rioux S., et al. 2010. Mass spectrometry-based metabolomics application to identify quantitative resistance-related metabolites in barley against *Fusarium head blight*. Mol. Plant Pathol. 11, 769-782.

Borges F., Martienssen R.A. 2015. The expanding world of small RNAs in plants. Nat. Rev. Mol. Cell Biol. 16, 727-741.

Brodersen P., Sakvarelidze-Achard L., Bruun-Rasmussen M., Dunoyer P., Yamamoto Y.Y., Sieburth L., Voinnet O. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. Sci. 30, 1185-1190.

Buer C.S., Imin N., Djordjevic M.A. 2010. Flavonoids: new roles for old molecules. J. Integr. Plant Biol. 52, 98-111.

Buer C.S., Muday G.K., Djordjevic M.A. 2007. Flavonoids are differentially taken up and transported long distances in Arabidopsis. Plant Physiol. 145, 478-490.

Casacuberta J.M., Raventós D., Puigdoménech P., San Segundo B. 1992. Expression of the gene encoding the *PR-like* protein *PRms* in germinating maize embryos. Mol. Gen. Genet. 234, 97-104.

Cavallarin L., Andreu D., San Segundo B. 1998. Cecropin A-derived peptides are potent inhibitors of fungal plant pathogens. Mol. Plant Microbe Interact. 11, 218–227.

Chen X.M. 2009. Small RNAs and their roles in plant development. Cell Dev. Biol. 25, 21–44.

Chiou T.J., Aung K., Lin S.I., Wu C.C., Chiang S.F., Su C.L. 2006. Regulation of phosphate homeostasis by MicroRNA in Arabidopsis. Plant Cell. 18, 412-421.

Clough S.J., Bent A.F. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. 6, 735-743.

Coego A., Ramirez V., Gil M.J., Flors V., Mauch-Mani B., Vera P. 2005. An Arabidopsis homeodomain transcription factor, *OVEREXPRESSOR OF CATIONIC PEROXIDASE 3*, mediates resistance to infection by necrotrophic pathogens. Plant Cell. 17, 2123-2137.

Conrath U. 2011. Molecular aspects of defence priming. Trends Plant Sci. 16, 524-531.

Conrath U., Beckers G.J., Langenbach C.J., Jaskiewicz M.R. 2015. Priming for enhanced defense. Ann. Rev. Phytopathol. 53, 97-119.

Dai G.H., Nicole M., Andary C., Martinez C., Bresson E., Boher B., et al. 1996. Flavonoids accumulate in cell walls, middle *lamellae* and callose-rich papillae during an incompatible interaction between *Xanthomonas campestris* pv. *malvacearum* and cotton. Physiol. and Mol. Plant Pathol. 49, 285-306.

De Conti Lourenço R.M., da Silva Melo P., Albino de Almeida A.B. 2013. Flavonoids as Antifungal Agents. Antifungal Metabolites from Plants. 283-300. DOI:10.1007/978-3-642-38076-1_10.

Denancé N., Sánchez-Vallet A., Goffner D., Molina A. 2013. Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. Front. Plant Sci. 4:155.

Dubos C., Stracke R., Grotewold E., Weisshaar B., Martin C., Lepiniec L. 2010. MYB transcription factors in Arabidopsis. Trends Plant Sci. 15, 573-581.

Fahlgren N.,Howell M.D.,Kasschau K.D., Chapman E.J., et al. 2007. High-Throughput Sequencing of Arabidopsis microRNAs: Evidence for Frequent Birth and Death of MIRNA Genes. PLoS ONE. 2:e219.

Falcone-Ferreyra M.L., Rius S.P., Casati P. 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front. Plant Sci. 3:222.

Foster-Hartnett D., Danesh D., Peñuela S., Sharopova N., Endre G., Vandenbosch K.A., et al. 2007. Molecular and cytological responses of *Medicago truncatula* to *Erysiphe pisi*. Mol. Plant Pathol. 8, 307–319.

Franco-Zorrilla J.M., Valli A., Todesco M., Mateos I., Puga M.I., Rubio-Somoza I., et al. 2007. *Target mimicry* provides a new mechanism for regulation of microRNA activity. Nat. Genet. 39, 1033-1037.

Galeottia F., Barileb E., Curirc P., Dolcia M., Lanzotti V. 2008. Flavonoids from carnation (*Dianthus caryophyllus*) and their antifungal activity. Phytochem. Letters 1, 44–48.

Gamir J., Pastor V., Kaever A., Cerezo M., Flors V. 2014. Targeting novel chemical and constitutive primed metabolites against *Plectosphaerella cucumerina*. Plant J. 78, 227-240.

Gao F., Wang N., Li H., Liu J., Fu C., Xiao Z., et al. 2016. Identification of drought-responsive microRNAs and their targets in *Ammopiptanthus mongolicus* by using high-throughput sequencing. Scientific Rep. 6:34601.

Glazebrook J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Ann. Rev. Phytopathol. 43, 205-227.

Huang J., Gu M., Lai Z., Fan B., Shi K., Zhou Y.H., et al. 2010. Functional analysis of the Arabidopsis *PAL* gene family in plant growth, development, and response to environmental stress. Plant Physiol.153, 1526-1538.

Huang J., Yang M., Zhang X. 2016. The function of small RNAs in plant biotic stress response. J. Integr. Plant Biol. 58, 312-327.

Iqbal M., Yaegashi S., Ahsan R., Shopinski K., Lightfoot D. 2005. Root response to *Fusarium solani* f. sp. glycines: temporal accumulation of transcripts in partially resistant and susceptible soybean. Theor. Appl. Genet. 110, 1429–1438.

Jefferson R.A., Kavanagh T.A., Bevan M.W., Kavanagh T.A., Bevan M.W. 1987. GUS fusions: *beta-glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907.

Jeong D.H., Green P.J. 2013. The role of rice microRNAs in abiotic stress responses. J. Plant Biol. 56, 187–197.

Jones J.D.G., Dangl J.L. 2006. The plant immune system. Nature. 444, 323–329.

Katiyar-Agarwal S., Jin H. 2010. Role of small RNAs in host-microbe interactions. Ann. Rev. Phytopathol. 48, 225-246.

König S., Feussner K., Kaever A., Landesfeind M., Thurow C., Karlovsky P., et al. 2014. Soluble phenylpropanoids are involved in the defense response of Arabidopsis against *Verticillium longisporum*. New Phytol. 202, 823-837.

Kuan T., Zhai Y., Ma W. 2016. Small RNAs regulate plant responses to filamentous pathogens. Semin. Cell Dev. Biol. 56, 190-200.

Lawton K.A., Potter S.L., Uknes S., Ryals J. 1994. Acquired resistance signal transduction in Arabidopsis is ethylene independent. Plant Cell 6, 581–590.

Lewis D.R., Ramirez M.V., Miller N.D., Vallabhaneni P., Ray W.K., Helm R.F., et al. 2011. Auxin and ethylene induce flavonol accumulation through distinct transcriptional networks. Plant Physiol. 156, 144-164.

Liu J., Osbourn A., Ma P. 2015. *MYB* Transcription Factors as Regulators of Phenylpropanoid Metabolism in Plants. Mol. Plant. 8, 689-708.

Llave C., Xie Z., Kasschau K. D., and Carrington J. C. 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science. 297, 2053–2056.

Llorente F., Alonso-Blanco C., Sánchez-Rodriguez C., Jorda L., Molina A. 2005. ERECTA receptor-like kinase and heterotrimeric G protein from Arabidopsis are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. Plant J. 43, 165-180.

Malamy J.E., Ryan K.S. 2001. Environmental regulation of lateral root initiation in Arabidopsis. Plant Physiol. 127, 899-909.

Martinez-Medina A., Flors V., Heil M., Mauch-Mani B., Pieterse C.M., Pozo M.J., et al. 2016. Recognizing Plant Defense Priming. Trends Plant Sci. 21,818-822.

Mauch-Mani and Slusarenko. 1994. Systemic acquired resistance in *Arabidopsis thaliana* induced by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. MPMI. 7, 378-383

Mehrtens F., Kranz H., Bednarek P., Weisshaar B. 2005. The Arabidopsis transcription factor *MYB12* is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol. 138, 1083-1096.

Miedes E., Vanholme R., Boerjan W., Molina A. 2014. The role of the secondary cell wall in plant resistance to pathogens. Front Plant Sci. 5:358.

Mierziak J., Kostyn K., Kulma A. 2014. Flavonoids as Important Molecules of Plant Interactions with the Environment. Molecules. 19, 16240-16265.

Molina A., Goy P.A., Fraile A., Sánchez-Monge R., García-Olmedo F. 1993a. Inhibition of bacterial and fungal plant pathogens by thionins of types I and II. Plant Sci. 92, 169-177.

Molina A., Segura A., García-Olmedo F. 1993b. Lipid transfer proteins (*nsLTPs*) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. FEBS Lett. 316, 119-220.

Mouradov A., Spangenberg G. 2014. Flavonoids: a metabolic network mediating plants adaptation to their real estate. Front. Plant Sci. 10, 5:620.

Navarro L., Dunoyer P., Jay F., Arnold B., Dharmasiri N., Estelle M., et al. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Sci. 312, 436-439.

O'Connell R.J., Herbert C., Sreenivasaprasad S., Khatib M., Esquerré-Tugayé M-T., Dumas B. 2004. A Novel *Arabidopsis–Colletotrichum* Pathosystem for the Molecular Dissection of Plant–Fungal Interactions. MPMI. 17, 272–282.

O'Connell R.J., Thon M.R., Hacquard S., Amyotte S.G., Kleemann J., Torres M.F., et al. 2012. Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. Nat. Genet. 44, 1060-1065.

Owens D.K., Alerding A.B., Crosby K.C., Bandara A.B., Westwood J.H., Winkel B.S. 2008. Functional analysis of a predicted flavonol synthase gene family in Arabidopsis. Plant Physiol. 147, 1046-1061.

Palatnik J.F., Allen E., Wu X., Schommer C., Schwab R., Carrington J.C., Weigel D. 2003. Control of leaf morphogenesis by microRNAs. Nature. 18; 257-263.

Palm M.E., Gams W., Nirenberg H.I. 1995. *Plectosporium*, a new genus for *Fusarium tabacinum*, the anamorph of Plectosphaerella cucumerina. Mycologia. 87, 397-406.

Peer W.A., Brown D.E., Tague B.W., Muday G.K., Taiz L., Murphy A.S. 2001. Flavonoid accumulation patterns of transparent testa mutants of arabidopsis. Plant Physiol. 126, 536-548.

Penninckx I.A., Thomma B.P., Buchala A., Métraux J.P., Broekaert W.F. 1998. Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell. 10, 2103-2113.

Preuss A., Stracke R., Weisshaar B., Hillebrecht A., Matern U., Martens S. 2009. *Arabidopsis thaliana* expresses a second functional flavonol synthase. FEBS Lett. 583, 1981–1986.

Rubio-Somoza I., Weigel D. 2011. MicroRNA networks and developmental plasticity in plants. Trends Plant Sci. 16, 258–264.

Sánchez-Vallet A., López G., Ramos B., Delgado-Cerezo M., Riviere M.P., Llorente F., et al. 2012. Disruption of abscisic acid signaling constitutively activates Arabidopsis resistance to the Necrotrophic fungus *Plectosphaerella cucumerina* Plant Physiol. 160, 2109-24.

Sánchez-Vallet A., Ramos B., Bednarek P., López G., Piślewska-Bednarek M., Schulze-Lefert P., Molina A. 2010. Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. Plant J. 63, 115-127.

Saslowsky D., Winkel-Shirley B. 2001. Localization of flavonoid enzymes in Arabidopsis roots. Plant J. 27, 37–48.

Seo J.K., Wu J., Lii Y., Li Y., Jin H. 2013. Contribution of small RNA pathway components in plant immunity. Mol. Plant Microbe Interact. 26, 617–625.

Sharma D., Tiwari M., Pandey A., Bhatia C., Sharma A., Trivedi P.K. 2016. MicroRNA858 Is a Potential Regulator of Phenylpropanoid Pathway and Plant Development. Plant Physiol. 171, 944-959.

Song S., Huang H., Gao H., Wang J., Wu D., Liu X., et al. 2014. Interaction between *MYC2* and *ETHYLENE INSENSITIVE3* modulates antagonism between jasmonate and ethylene signaling in Arabidopsis. Plant Cell. 26, 263-279.

Staiger D., Korneli C., Lummer M., Navarro L. 2013. Emerging role for RNA-based regulation in plant immunity. New Phytol. 197, 394–404.

Stracke R., Ishihara H.,Huep G., Barsch A., Mehrtens F.,Niehaus K., Weisshaar B. 2007. Differential regulation of closely related *R2R3-MYB* transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J. 50, 660–677.

Tao Y., Xie Z., Chen W., Glazebrook J., Chang H.S., Han B., et al. 2003. Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. Plant Cell. 15, 317-330.

Thomma B.P., Eggermont K., Penninckx I.A., Mauch-Mani B., Vogelsang R., Cammue B.P., Broekaert W.F. 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA. 95, 15107-15111.

Thomma B.P., Eggermont K., Tierens K.F., Broekaert W.F. 1999. Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiol. 121, 1093-1102.

Todesco M., Rubio-Somoza I., Paz-Ares J., Weigel D. 2010. A collection of target mimics for comprehensive analysis of microRNA function in *Arabidopsis thaliana*. PLoS Genet 6:e1001031.

van Loon L.C., Rep M., Pieterse C.M. 2006. Significance of inducible defense-related proteins in infected plants. Ann. Rev. Phytopathol. 44, 135-162.

Vogt T. 2010. Phenylpropanoid biosynthesis. Mol. Plant. 3, 2-20.

Watkins J.M., Hechler P.J., Muday G.K. 2014. Ethylene-induced flavonol accumulation in guard cells suppresses reactive oxygen species and moderates stomatal aperture. Plant Physiol. 164, 1707-1717.

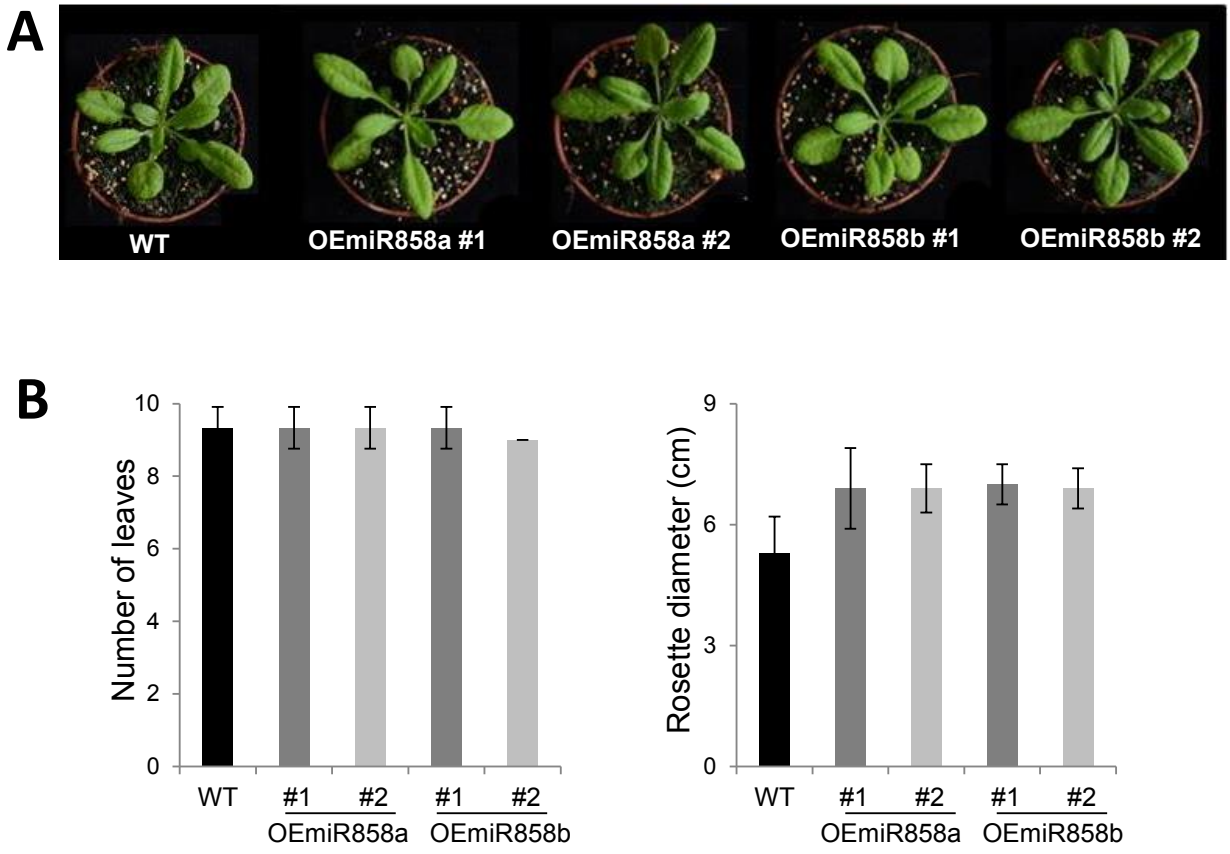
Weiberg A., Wang M., Bellinger M., Jin H. 2014. Small RNAs: a new paradigm in plant-microbe interactions. Ann. Rev. Phytopathol. 52, 495-516.

Xia R., Zhu H., An Y.Q., Beers E.P., Liu Z. 2012. Apple miRNAs and tasiRNAs with novel regulatory networks. Genome Biol. 13:R47.

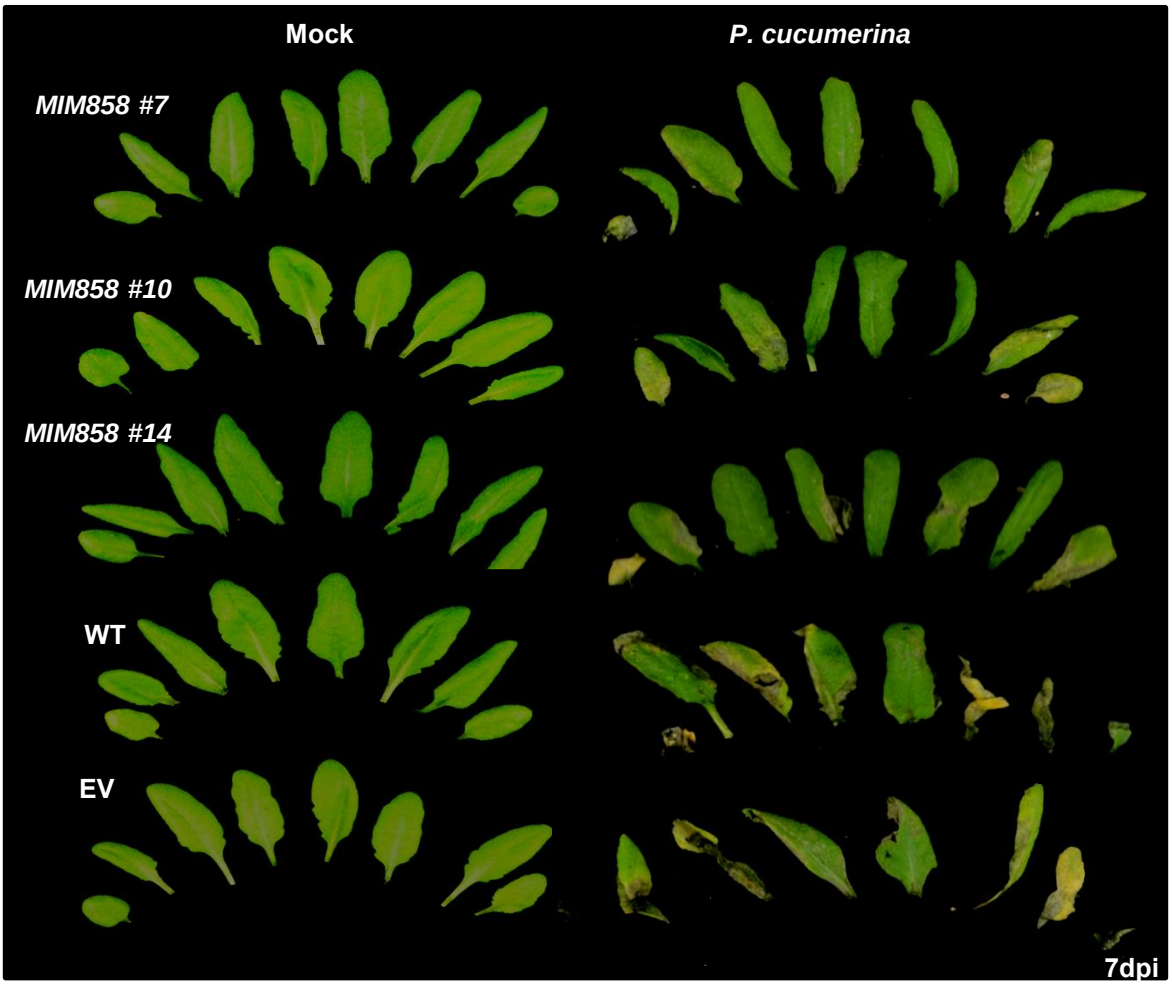
Xu W., Dubos C., Lepiniec L. 2015. Transcriptional control of flavonoid biosynthesis by *MYB-bHLH-WDR* complexes. Trends Plant Sci. 20, 176-185.

Yang L., Huang H. 2014. Roles of small RNAs in plant disease resistance. J. Integr. Plant Biol. 56, 962-970.

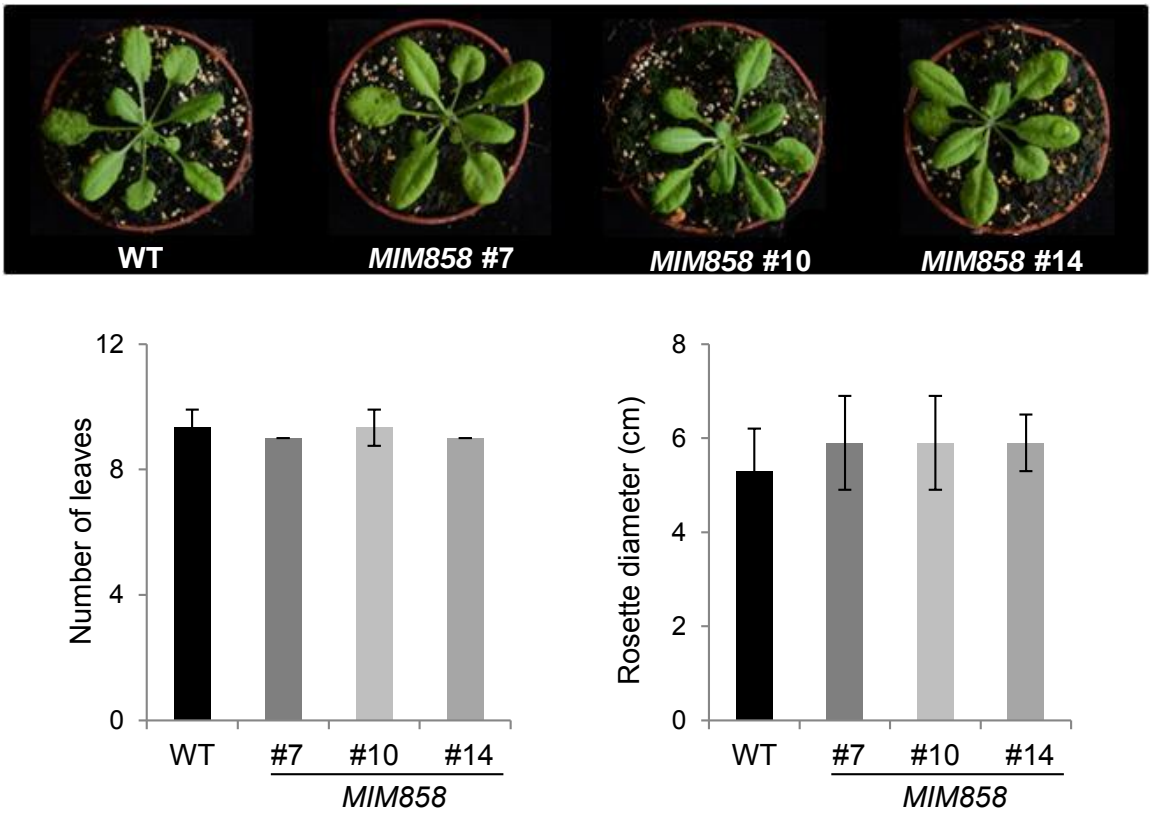
Zipfel C. 2014. Plant pattern-recognition receptors. Trends Immunol. 35, 345-351.



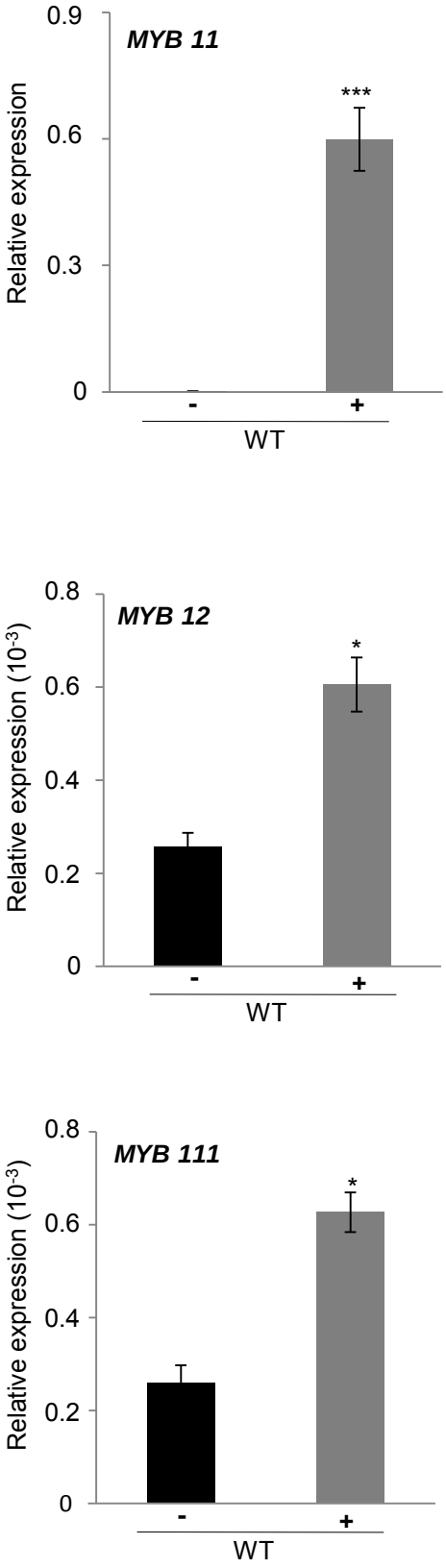
Supplemental Figure S1. Phenotype of miR858 overexpressor (OE miR858a, OE miR858b) plants grown for 3 weeks under a 12h light/12h dark photoperiod condition. Two independent experiments were carried out with 3 independent *MIM858* lines (at least 24 plants per line, each experiment). **(A)** Rosette phenotypes of wild-type, OE miR858a and OE miR858b plants. **(B)** Rosette leaf number and diameter (left and right panel, respectively). No significant differences were observed in leaf number between wild-type, *MIM858* and wild type plants (ANOVA test). The rosette diameter (cm) was determined using Image J software (right panel).



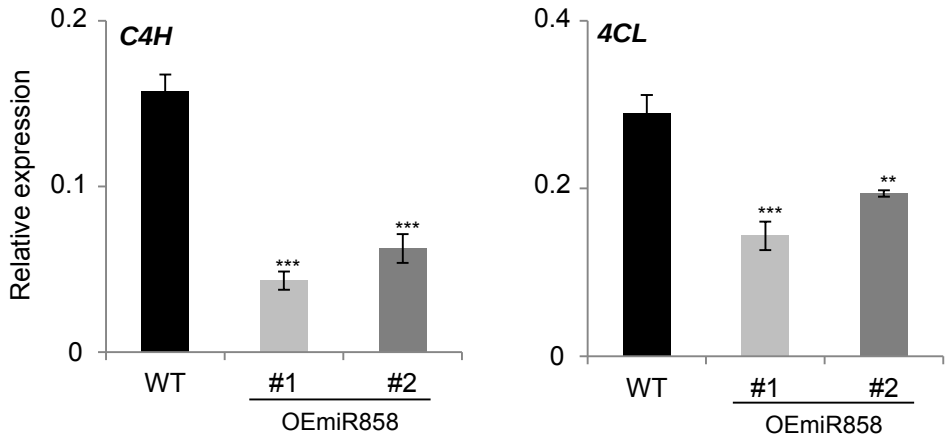
Supplemental Figure S2. Dissected leaves of three-week-old *MIM858* plants before inoculation (left) and 7 days after inoculation with *P. cucumerina* spores (4 x 10^6 spores/ml).



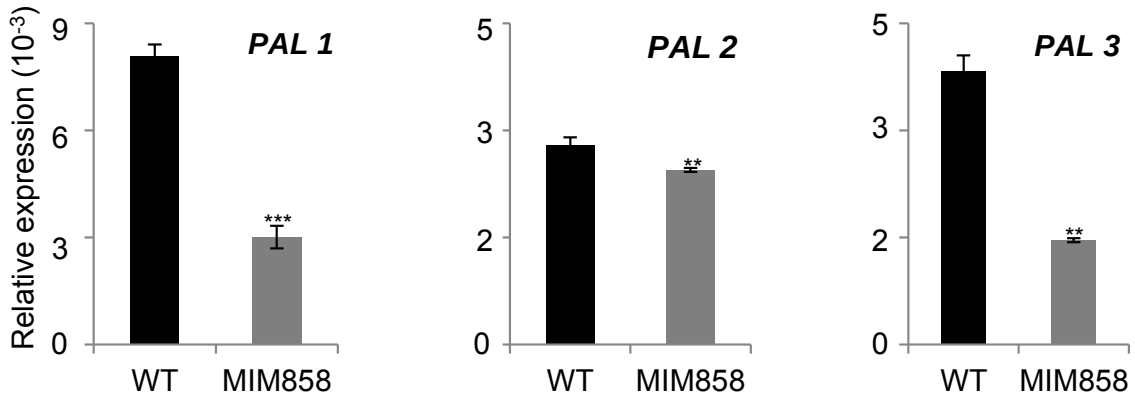
Supplemental Figure S3 Phenotype of *MIM858* plants grown for 3 weeks under a 12h light/12h dark photoperiod condition. Two independent experiments were carried out with 3 independent *MIM858* lines (at least 24 plants per line, each experiment). **(A)** Rosette phenotypes of wild-type and *MIM858* plants. **(B)** Rosette leaf number and diameter (left and right panel, respectively). No significant differences were observed in leaf number between wild-type, *MIM858* and wild type plants (ANOVA test). The rosette diameter (cm) was determined using Image J software (right panel).



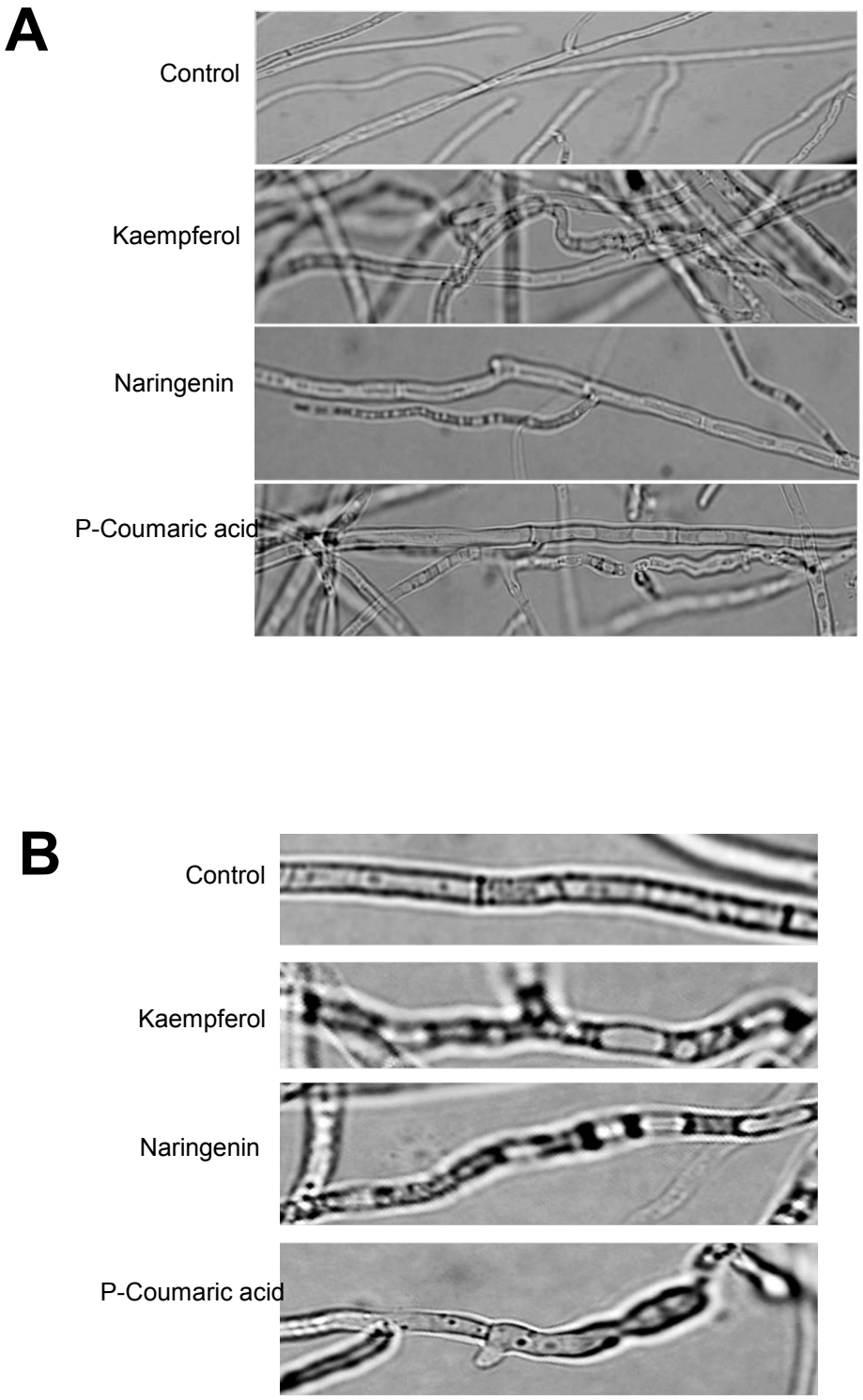
Supplemental Figure S4. Expression of *MYB* genes that are regulated by miR858 in wild type plants under control (-, non-infected) and after inoculation with *P. cucumerina* (72hpi). RT-qPCR analysis was carried out using the *β-tubulin2* gene (*At5g62690*) as the internal control. Statistical significance was determined by ANOVA (*, P ≤ 0.05; ***, P ≤ 0.001). Histograms show the mean ± SD of 2 biological replicates, each with 24 plants per genotype.



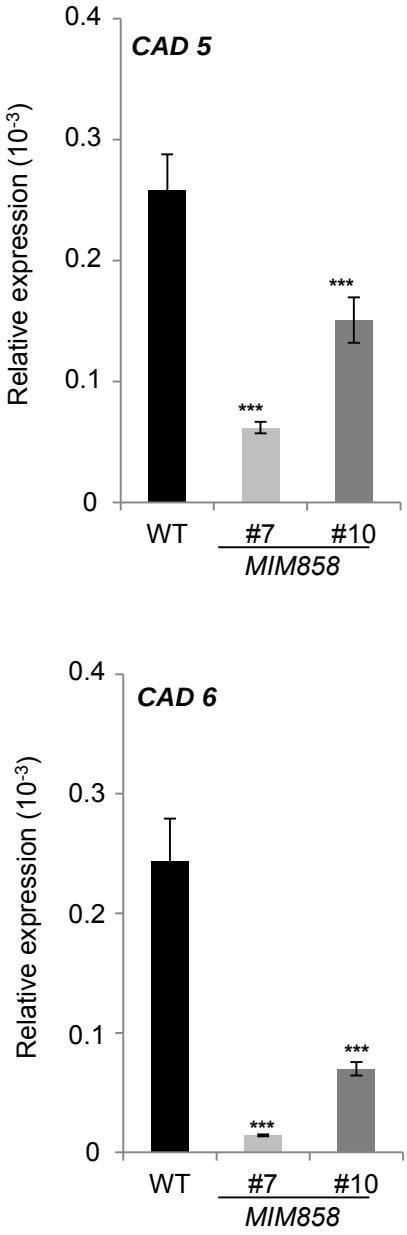
Supplemental Figure S5. RT-qPCR analysis of *C4H* and *4CL* in OE miR858 plants. The *β-tubulin2* gene (*At5g62690*) was used as the internal control. Histograms show the mean ± SD of 2 biological replicates, each with 24 plants per genotype. Statistical significance was determined by ANOVA (**, P ≤ 0.01; ***, P ≤ 0.001).



Supplemental Figure S6. RT-qPCR analysis of *PAL1, PAL2* and PAL3 in *MIM858* plants. The *β-tubulin2* gene (*At5g62690*) was used as the internal control. Statistical significance was determined by ANOVA (**, P ≤ 0.01; ***, P ≤ 0.001).



Supplemental Figure S7. Differences in morphology of hyphae in *P. cucumerina* cultures grown in PDB medium in the presence of naringenin, kaempferol or p-Coumaric acid at the indicated concentrations. Micrographs were taken after 48 h of incubation. Fungal growth is expressed as the percentage of the growth of control cultures, without flavonoid/phenypropanoid compound). Bioassays were carried out 3 times with different preparations of spore suspensions.



Supplemental Figure S8. RT-qPCR analysis of CAD5, CAD6 expression in *MIM858* plants. The *β-tubulin2* gene (*At5g62690*) was used as the internal control. Histograms show the mean ± SD of 2 biological replicates, each with 24 plants per genotype. Statistical significance was determined by ANOVA (**, P ≤ 0.01; ***, P ≤ 0.001).

Supplemental Table 1. Sequences of oligonucleotides used in this study.			
Name	**Accesion number**	**Sequence (5'-3')**	**Use**
MYB11 Fw	*At4g09460*	CCGGGAAGAACAGACAACGA	RT-qPCR
MYB11 Rv		TTCTCGACGGTATTGGCGAC	
MYB12 Fw	*At2g47460*	GGTGGTCACTAATCGCGGGT	RT-qPCR
MYB12 Rv		GCGTTCGTCATGATTACGGCG	
MYB13 Fw	*At1g06180*	TCAACTCTTAGGCAACAGATGG	RT-qPCR
MYB13 Rv		TGATCTTGACTGTGGTGGAGTC	
MYB20 Fw	*At1g66230*	TGGAAACAGGTGGTCAAAGATA	RT-qPCR
MYB20 Rv		ATCCCCATTTTCCTCAACTTCT	
MYB111 Fw	*At5g49330*	GCATTCCCTTCTCGGCAACAGAT	RT-qPCR
MYB111 Rv		CGTTGACGAGTAGATTGTGTCC	
PDF1.2 Fw	*At2g26020*	CAACAATGGTGGAAGCACAG	RT-qPCR
PDF1.2 Rv		CTTGCATGCATTGCTGTTTC	
PR4 Fw	*At3g04720*	TGTGAGAATAGTGGACCAATGC	RT-qPCR
PR4 Rv		CCATCGGTGTCTATTTGATTGA	
VSP2 Fw	*At5g24770*	CTCGTCGATTCGAAAACCAT	RT-qPCR
VSP2 Rv		TTCTGCAGTTGGCGTAGTTG	
LOX2 Fw	*At3g45140*	ATCAACAAGCCCCAATGGAA	RT-qPCR
LOX2 Rv		CGGCGTCATGAGAGATAGCAT	
PR1a Fw	*At2g14610*	GATGTGCCAAAGTGAGGTGTAA	RT-qPCR
PR1a Rv		GGCTTCTCGTTCACATAATTCC	
NPR1 Fw	*At1g64280*	CCGGAAGAGCTTGTTAAAGAGA	RT-qPCR
NPR1 Rv		ATCCGAGTCAAGTGCCTTATGT	
ACS Fw	*At3g61510*	ACGCTTTTCTCGTCCCTACTC	RT-qPCR
ACS Rv		GGCCTTAAGGTACGCTGATTC	
ACO2 Fw	*At1g62380*	AGGAAACAGGATGTCGGTTG	RT-qPCR
ACO2 Rv		CGGAATCTTTCTCGACAAGC	
PAL1 Fw	*At2g37040*	GTGTCGCACTTCAGAAGGAA	RT-qPCR
PAL1 Rv		GGCTTGTTTCTTTCGTGCTT	
PAL2 Fw	*At3g53260*	GTGCTACTTCTCACCGGAGA	RT-qPCR
PAL2 Rv		TATTCCGGCGTTCAAAAATC	
PAL3 Fw	*At5g04230*	CAACCAAACGCAACAGCA	RT-qPCR
PAL3 Rv		CTCCAGGTGGCTCCCTTTTA	
PAL4 Fw	*At3g10340*	GGTGCACTTCAAAATGAGCT	RT-qPCR
PAL4 Rv		CAACGTGTGTGACGTGTCC	
C4H Fw	At2g30490	TGAGTTTGGATCCAGAACGAG	RT-qPCR
C4H Rv		CGTCATGATTCTTCTCATCTTCCT	
4CL1 Fw	*At1g51680*	TCAACCCGGTGAGATTTGTA	RT-qPCR
4CL1 Rv		TCGTCATCGATCAATCCAAT	
CAD5 Fw	*At4g34230*	TGATCTTGGCATGTCTAATTACC	RT-qPCR
CAD5 Rv		GACATCAACGACGAACCTAT	
CAD6 Fw	*At4g37970*	GAGCGTAGAGGCGTTTGG	RT-qPCR
CAD6 Rv		CGAAACTAGAGGCAATGTGT	
β-Tubulin2 Fw	*At5g62690*	TGTTCAGGCGAGTGAGTGAG	RT-qPCR
β-Tubulin2 Rv		ATGTTGCTCTCCGCTTCTGT	
PC_tubulin Fw	-	CAAGTAtGTTCCCCGAGCCGT	qPCR (*P. cucumerina*)
PC_tubulin Rv		GAAGAGCTGACCGAAGGGACC	
FOC_chsV Fw	-	ACAGCTCCAACGAACTCTCTT	qPCR (*F. oxysporum*)
FOC_chsV Rv		GGAGGTACTTGGTCAtGTCGT	
Ch_ITS2 Fw	-	AAAGGTAGTGGCGGACCCTC	qPCR (*C. higginsianum*)
Ch_ITS2 Rv		GGCAAGAGTCCCTCCGGAt	
Ubiquitin21 Fw	*At5g25760*	AAAGGACCTTCGGAGACTCCTTACG	qPCR (fungal biomass)
Ubiquitin21 Rv		GGTCAAGAAtCGAACTTGAGGAGGTT	
miR858a Stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGTCGA		stem-loop RT-PCR
	TTTCGTTGTCTGTTCG		
miR858b Stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGGTC		stem loop RT-PCR
	TTCGTTGTCTGTTC		
pre-miR858a Fw	CGCGCTTTATCGTTTATTTCA		RT-PCR
pre-miR858a Rv	TACCCCAATCCCCATCAATA		
pre-miR858b Fw	TGGTTTGGTTTTGGGTTTTG		RT-PCR
pre-miR858b Rv	GGAAGATCGAGGCATATGGA		

Supplemental Table S1. Sequences of oligonucleotides used in this study.

GENERAL DISCUSSION

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Los miARNs juegan un papel importante en la regulación de la expresión génica en eucariotas. En plantas, los miARNs participan en varios procesos asociados al desarrollo y señalización mediada por hormonas. Algunos miARNs también se han visto involucrados en las respuestas de las plantas frente a estreses bióticos y abióticos, estrés oxidativo y la deficiencia de nutrientes. En el arroz, la actividad de ciertos miARNs controla caracteres de importancia agronómica, p.e. la floración temprana, el desarrollo de la panícula y la producción de granos. Sin embargo, se conoce menos sobre la función de los miARNs en las interacciones planta-patógeno.

Este trabajo se ha centrado en la identificación y validación funcional de miARNs en la respuesta de las plantas frente a la infección por patógenos, concretamente patógenos fúngicos. Estos estudios se han llevado a cabo en Arabidopsis y arroz, los modelos utilizados en genómica funcional en plantas dicotiledóneas y monocotiledóneas, respectivamente. Los resultados obtenidos en la presente Tesis Doctoral demuestran que los miARNs pueden desempeñar una función importante en la respuesta de defensa de las plantas frente a la infección por hongos patógenos.

Para el estudio de miARNs de arroz, se han empleado herramientas bioinformáticas para la predicción de la estructura secundaria de precursores de miARN y para la predicción de posibles genes diana de miARNs. Inicialmente, se llevó a cabo un análisis de las secuencias de ARN previamente identificadas por secuenciación masiva de poblaciones de pequeños ARNs con el objeto de identificar aquellas secuencias que mapean en regiones del genoma de arroz que generan tránscritos con potencial formar estructuras en horquilla (característica de precursores de ARN). De esta manera se seleccionaron 5 miARNs candidatos que podían representar nuevos miARNs de arroz: miR-64, miR-75, miR-96, miR-98 y miR-203. Para la validación de estas secuencias como nuevos miARNs se ha tenido en cuenta los criterios utilizados para la anotación de nuevos miARNs en el repositorio oficial de miARN (miRBase; http://www.mirbase.org/). Es de destacar el hecho de que a pesar del bajo nivel de acumulación de los pequeños ARNs aquí estudiados, se ha podido demostrar la presencia de las dos cadenas del dúplex (miRNA-5p, miRNA-3p) para los 5 candidatos estudiados. Ello, junto con los resultados obtenidos en el análisis de mutantes afectados en la biosíntesis de miARNs (mutantes *dcl1*, *dcl2* y *dcl3*) refuerzan la idea de que miR-75, miR-64 y miR-96 son

realmente miARNs de arroz. Además, los resultados obtenidos en mutantes afectados en la biogénesis de miARNs, mostraron que muy probablemente estos miARNs son de reciente aparición en la escala evolutiva. El hecho de que únicamente 2 de los 5 miARNs aquí estudiados, hayan sido identificados en otra especie vegetal refuerza esta idea. Así, miR-64 se encuentra en *Theobroma cacao* (cacao) y miR-203 en la palma africana aceitera (*Elaeis guineensis*).

La prueba definitiva de la implicación de los miARNs (o miARNs candidatos) en la respuesta inmune de la planta de arroz se obtuvo en plantas transgénicas que sobreexpresan el miARN estudiado. De esta manera, se ha podido demostrar que la sobreexpresión de miR-75 o miR-64 confiere resistencia frente a la infección por *M. oryzae*, mientras que la sobreexpresión de miR-96, miR-98 o miR-203 aumentan la susceptibilidad de la planta. Estos resultados indican que miR-75 y miR-64 regulan positivamente la inmunidad innata, mientras que miR-96, miR-98 y miR-203 son reguladores negativos de la respuesta inmune en arroz. El fenotipo observado de resistencia o susceptibilidad en las plantas transgénicas se correlaciona con la intensidad en la respuesta a la infección del gen *OsPR1b* (marcador de la inducción de la respuesta de defensa). Así, las plantas que sobreexpresan miR-64 o miR-75 muestran una fuerte activación de *OsPR1b* durante la infección por *M. oryzae*, mientras que plantas que sobreexpresan miR-96, miR-98 y miR-203 tuvieron una menor induccion de la expresión de *OsPR1b* comparadas con plantas control. En cualquier caso, es necesario llevar a cabo un investigación más detallada para determinar el efecto de la sobreexpresión de cada uno de estos miARNs candidatos en relación a la expresión de genes de defensa de arroz, y poder de esta manera determinar los procesos que se encuentran regulados por estos miARNs candidatos.

En el caso concreto de miR-75, la búsqueda en el degradoma de arroz permitió demostrar que este miARN degrada tráncritos de una β-1,3-glucanasa, por lo que se puede pensar que miR-75 regula la expresión de este gen durante la infección. Las β-1,3-glucanasas son proteínas ampliamente distribuidas en plantas con una función asociada a la defensa frente a patógenos (hongos) (Ye et al., 1990; Arlorio et al., 1992). Las β-1,3-glucanasas junto con las chitinasas hidrolizan β-1,3-glucanos y chitina, respectivamente, que son los componentes de la pared celular de muchos hongos patogénicos. De esta manera, la actividad de β-1,3-glucanasas permite la liberación de oligosacáridos de las paredes celulares del patógeno que pueden luego actúar como

elicitores de la respuesta de defensa de la planta (Bowles, 1990; Meins et al., 1992 Velazhahan et al., 2000, Van Loon et al., 2006). Estudios en mutantes deficientes para β-1,3-glucanasas han mostrado que estos enzimas pueden asimismo participar en la defensa frente a virus. Se piensa que esta función se basa en la regulación de los depósitos de callosa (polímero de β-1,3-glucano) en el cuello de los plasmodesmos. Los plasmodesmos son canales que conectan el citoplasma de las células vegetales vecinas, y también la vía que utilizan los virus para su transmisión célula-a-célula. Además de su función en defensa, existen también evidencias de que estos enzimas están involucrados en diferentes procesos del desarrollo reproductivo (germinación del polen y crecimiento del tubo polinico) (Bucciaglia and Smith, 1994; Leubner-Metzger, 2003; Zavaliev et al., 2010).

En su conjunto, los resultados presentados en los capítulos I y II de la presente tesis demuestran que los miARNs estudiados en este trabajo pueden ser considerados una parte importante en la respuesta inmune de la planta de arroz. Ello viene a reforzar la idea de que los miARNs desempeñan un papel importante como reguladores de la expresión génica en la inmunidad innata de las plantas. Si bien se ha podido demostrar la funcionalidad de los miARN/miARNs candidatos aquí estudiados, todavía quedan muchos aspectos por aclarar en relación a los procesos concretos en los que estos miARNs participan y como estos procesos interaccionan con otras vías de señalización implicadas en respuesta de defensa de la planta.

Durante el transcurso de la presente tesis, se han preparado construcciones para el silenciamiento de los otros 4 miARNs/miARN candidatos mediante el sistema CRISPR/Cas9 (Miao, et al., 2013). Se pretende así poder corroborar los resultados obtenidos en plantas transgénicas que sobreexpresan dichos miARNs/miARN candidatos (trabajo en curso). Además, dadas las dificultades que existen para disponer de mutantes con expresión silenciada (mutantes de inserción de T-DNA) de miARNs, resulta de especial interés poder contar con una herramienta válida para el silenciamiento de genes *MIR*, como es el caso del sistema CRISPR/Cas9. En nuestro caso, esta tecnología ha demostrado ser útil para el silenciamiento de *MIR-75*.

Para concluir, teniendo en cuenta que el arroz es uno de los cereales más importantes destinado al consumo humano, y que la enfermedad de la piriculariosis causada por el hongo *M. oryzae* genera importantes pérdidas económicas en el cultivo del arroz a nivel mundial, el esclarecimiento de la función de miARNs, de sus genes diana, y de los

procesos que son regulados por ellos, representa la base de partida para el desarrollo de nuevas estrategias para la protección del arroz frente a enfermedades.

En el capítulo III de la presente Tesis Doctoral, se ha demostrado que miR858 es un componente de la inmunidad innata de la planta de Arabidopsis en su interacción con hongos patógenos. Las plantas que sobreexpresan miARN demostraron ser más susceptibles a la infección por patógenos fúngicos que las plantas salvajes, mientras que líneas transgénicas de imitación de diana (líneas *MIM858*, amablemente cedidas por los Drs I. Rubio & D. Weigel) mostraron un fenotipo de resistencia a la infección. La resistencia en las plantas *MIM858* es efectiva frente al hongo necrótrofo *Plectosphaerella cucumerina* y a los hongos hemibiótrofos *Fusarium oxysporum* f. sp *conglutinans* y *Colletotrichum higginsianum*. El análisis de la expresión de genes de defensa asociados a la señalización por las hormonas implicadas en defensa, ET, JA y SA, mostró que en condiciones de infección los genes de defensa que son regulados por ET (*PDF1.2, PR4*), así como los genes clave en la biosíntesis de ET (*ACO, ACS*), muestran una mayor inducción de su expresión. Estos resultados sugieren que miR858 es un regulador negativo en las respuestas de defensa en Arabidopsis que son reguladas por ET. No está clara la conexión molecular entre miR858 (regulador de la expresión de factores de transcripción implicados en la biosíntesis de flavonoides) y la expresión de los genes de defensa.

En este trabajo se ha demostrado que la resistencia que se observa a la infección por hongos en las líneas *MIM858* puede ser el resultado de la actividad de miR858 sobre factores de transcripción de tipo MYB (*MYB11*, *MYB12* y *MYB111*), activadores transcripcionales de la expresión de genes de la biosíntesis de flavonoides en Arabidopsis (Stracke et al., 2007; Ravaglia et al., 2013; Zhang et al., 2015; Sharma et al., 2016). Ello conlleva la acumulación de flavonoides en hoja a expensas de la producción de monolignoles (unidades estructurales de la lignina). La observación de que los flavonoides kaempferol y quercetina, además del fenilpropanoide ácido p-cumárico poseen actividad antifúngica frente a *P. cucumerina* apoya la idea de que la mayor acumulación de estos metabolitos inhibe el crecimiento del hongo y colonicazión del tejido de la planta.

Como conclusión, los resultados obtenidos en esta Tesis Doctoral ponen de manifiesto que los miARNs desempeñan un papel importante en las respuestas de defensa en plantas frente a agentes patógenos. Dado que las enfermedades causadas por

patógenos son una de las causas más importantes de pérdidas en las cosechas, la información obtenida en estos estudios puede resultar de interés para poder alcanzar un mejor conocimiento de los procesos regulados miARNs en la inmunidad innata de las plantas. Ello podría a su ver permitir el diseño de nuevas estrategias para la protección frente a enfermedades en cultivos de importancia agronómica.

CONCLUSIONS

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1. **En arroz**, se han identificado 3 nuevos miARNs, miR-75, miR-64 and miR-96. El análisis de mutantes de arroz afectados en la biogénesis de miARNs mostró que la acumulación de estos miARNs es dependiente de DCL3 y/o DCL4. De ellos, únicamente miR-64 se encuentra en otra especie vegetal. Todo ello, sugiere que se trata de un miARN de reciente evolución. Además, se han identificado 2 pequeños ARNs candidatos a representar nuevos miARNs de arroz, miR-98 y miR-203.

2. La sobreexpresión de miR-75 y miR-64 confiere resistencia a la infección por el hongo *Magnaporthe oryzae* en arroz, lo que sugiere que estos miARNs son reguladores positivos de la respuesta de defensa de la planta.

3. Mediante el sistema de edición génica CRISPR/Cas9 se han obtenido mutantes de arroz con una delección en la secuencia del precursor miR-75. Estos mutantes son más susceptibles a la infección por *M. oryzae*, lo que concuerda con el fenotipo de resistencia observado en las plantas que sobreexpresan miR-75. Muy probablemente, miR-75 regula la expresión de un gen *β-1,3-glucanasa* cuya función está asociada a la defensa de las plantas frente a infección por hongos.

4. La sobreexpresión de miR-96, miR-98 y miR-203 en plantas de arroz aumenta la susceptibilidad a la infección por *M. oryzae*, indicando que estos miARNs/miARN candidatos regulan negativamente la respuesta de defensa de la planta de arroz.

5. **En Arabidopsis**, miR858 regula la expresión de factores de transcripción del tipo MYB, que funcionan como activadores transcripcionales de la expresión de genes para la biosíntesis de flavonoides. Durante la infección con el hongo necrótrofo *Plectosphaerella cucumerina,* la acumulación de miR858 se ve disminuida con el consiguiente aumento en la expresión de los factores de transcripción tipo MYB (*AtMYB11*, *AtMYB12*, *AtMYB111*).

6. La sobreexpresión de miR858 aumenta la susceptibilidad a infección por el hongo *P. cucumerina,* mientras que la interferencia con la actividad de este miARN en líneas de imitación de diana (líneas *MIM858*) confiere resistencia a la infección, no sólo del hongo necrótrofo *P. cucumerina* sino también de los hongos hemibiótrofos *Fusarium oxysporum f. sp. conglutinans* y *Colletrotrichum higginsianum*. Durante la infección, se observa una super-inducción de genes de defensa dependientes de etileno, así como también de genes para la biosíntesis de esta hormona. Muy probablemente, miR858 (o procesos regulados por este miARN), interaccionan con la ruta de transducción de la señal para la activación de las respuestas de defensa mediadas por etileno. Se desconoce el mecanismo por el cual miRNA podría estar regulando estas respuestas de defensa.

7. En plantas *MIM858*, la interferencia con la actividad miR858, y el consiguiente aumento en la expresión de sus genes *MYB* diana, conlleva un redireccionamiento en las ruta de biosíntesis de fenilpropanoides con una mayor acumulación de fenilpropanoides en hojas de plantas *MIM858*, a expensas de una disminución en la síntesis de monolignoles (unidades estructurales de la lignina).

8. Se ha demostrado la actividad antifúngica frente a *P. cucumerina* de los flavonoides, kaempferol y quercetina, así como también del fenilpropanoide ácido p-cumárico. Dado que las plantas *MIM858* acumulan flavonoides en sus hojas, se puede pensar que dicha acumulación puede ser un factor importante para la resistencia a infección que se observa en las plantas *MIM858*. El efecto protector se explicaría por la actividad antifúngica de flavonoides, a modo individual o en combinación con otros flavonoides y/o fenilpropanoides (p.e. ácido p-cumárico).

9. El conjunto de resultados obtenidos en este trabajo refuerzan la idea de que la regulación de la expresión génica mediada por miARNs participa de manera importante en la respuesta immune en arroz y Arabidopsis.

CONCLUSIONS

1. **In rice**, 3 novel miRNAs have been identified: miR-75, miR-64 and miR-96. The analysis of rice mutants affected in the biogenesis of miRNAs showed that the accumulation of these miRNAs is dependent on DCL3 and/or DCL4. Only miR-64 is found in another plant species. All this suggests that is a miRNA recently evolved. Additionally, it has been identified 2 small RNAs that represent novel miRNAs candidates of rice, miR-98 and miR-203.

2. Overexpression of miR-75 and miR-64 confers resistance to infection by *Magnaporthe oryzae* in rice, suggesting that these miRNAs are positive regulators of plant defense responses.

3. By means of the gene editing CRISPR/Cas9 system, rice mutants of miR-75 with genomic deletions in miR-75 precursor have been obtained. These mutants are more susceptible to infection by *M. oryzae*, in agreement with the resistance phenotype observed in miR-75 overexpresor plants. Probably, miR-75 regulates β-1,3-glucanase gene expression, whose function is associated to plant defense against fungal infection.

4. Overexpression of miR-96, miR-98 or miR-203 increases the susceptibility to infection by *M. oryzae* in rice plants, indicating that these miRNAs/miRNA candidates negatively regulate the defense response in rice.

5. **In Arabidopsis** miR858 negatively regulates MYB transcription factors expression, which functions as transcriptional activators of gene expression for the biosynthesis of flavonoids. During infection by necrotrophic fungus *Plectosphaerella cucumerina,* the miR858 accumulation decreased with the consequent increase in the expression of MYB transcription factors (*AtMYB11*, *AtMYB12*, *AtMYB111*).

6. Overexpression of miR858 increases susceptibility to infection by the fungus *P. cucumerina,* while the interference with the activity of this miRNA in target mimic

lines (*MIM858* plants) confers resistance to infection, not only to the necrotrophic fungus *P. cucumerina* but also to hemibiotrophic fungi *Fusarium oxysporum f. sp. conglutinans* and *Colletrotrichum higginsianum*. During the infection, a higher induction of ethylene-dependent defense genes, as well as genes for the biosynthesis of this hormone, was observed. Probably, miR858 (or processes regulated by this miRNA), interacts with the signal transduction pathway for the activation of ethylene-mediated defense responses. The mechanism by which miRNA might be regulating these defense responses is unknown.

7. In *MIM858* plants, interference with miR858 activity, and the consequent increase in the expression of their target *MYB* genes, carry a redirection in the phenylpropanoid biosynthetic pathways with a greater accumulation of phenylpropanoides in leaves of *MIM858* lines, at the expense of a decrease in the synthesis of monolignols (lignin structural units).

8. The antifungal activity against *P. cucumerina* of the flavonoids, kaempferol and quercetin, as well as of the phenylpropanoide p-Coumaric acid has been demonstrated. Since *MIM858* plants accumulate flavonoids in their leaves, it may be thought that such accumulation may be an important factor for the resistance to infection observed in *MIM858* plants. The protective effect would be explained by the antifungal activity of flavonoids, individually or in combination with other flavonoids and/or phenylpropanoids (i.e. p-Coumaric acid).

9. The set of results obtained in this work reinforces the idea that the regulation of gene expression mediated by miRNAs plays an important role in the immune response in rice and Arabidopsis.

BIBLIOGRAPHY

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Ahuja I., Kissen R., Bones A.M. 2012. Phytoalexins in defense against pathogens. Trends Plant Sci. 17, 73-90.

Albrecht C., Boutrot F., Segonzac C., Schwessinger B., Gimenez-Ibanez S., Chinchilla S., et al. 2012. Brassinosteroids inhibit pathogen-associated molecular pattern-triggered immune signaling independent of the receptor kinase BAK1.Proc. Natl. Acad. Sci. USA. 109, 303–308.

Allarangaye M.D., Traoré O., Traoré E.V.S., Millogo R.J., Konaté G. 2006. Evidence of non-transmission of RICE YELLOW MOTTLE VIRUS through seeds of wild host species. J. Plant Pathol. 88, 309-315.

Ambawat S., Sharma P., Yadav N.R., Yadav R.C. 2013. MYB transcription factor genes as regulators for plant responses: an overview. Physiol. Mol. Biol. Plants. 19, 307–321.

Arabidopsis T., Arabidopsis T., Initiative G., et al. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature. 408, 796–815.

Arikit S., Zhai J., Meyers B.C. 2013. Biogenesis and function of rice small RNAs from non-coding RNA precursors. Curr. Opin. Plant Biol. 16, 170-179.

Arlorio M., Ludwig A., Boiler T., Bonfonte P. 1992. Inhibition of fungal growth by plant chitinases and β-l,3-glucanases: a morphological study. Protoplasma171, 34–43.

Ashikawa I., Hayashi N., Yamane H., Kanamori H., Wu J., Matsumoto T., et al. 2008. Two adjacent nucleotide-binding site–leucine-rich repeat class genes are required to confer Pikm-specific rice blast resistance. Genet. 180, 2267–2276.

Axtell M.J. 2013. Classification and Comparison of Small RNAs from Plants. Ann. Rev. of Plant Biol. 64:1-863.

Baldrich P., San Segundo B. 2016. MicroRNAs in Rice Innate Immunity. Rice 9, 1–6.

Baldrich P., Campo S., Wu M-T., Liu T-T., Hsing Y-IC., San Segundo B. 2015. MicroRNA-mediated regulation of gene expression in the response of rice plants to fungal elicitors. RNA Biol. 12, 847–863.

Ballini E., Morel J.B., Droc G., Price A., Courtois B., Notteghem J.L., Tharreau D. 2008. A genomewide meta-analysis of rice blast resistance genes and quantitative trait loci provides new insights into partial and complete resistance. Mol. Plant Microbe Int. 21, 859–868.

Bari R., Jones J.D. 2009. Role of plant hormones in plant defence responses. Plant Mol Biol. 69, 473-488.

Baudouin E., Hancock J.T. 2014. Nitric oxide signaling in plants. Front. Plant Sci. 4:553.

Baxter A., Mittler R., Suzuki N. 2014. ROS as key players in plant stress signaling. J. Exp. Bot. doi: 10.1093/jxb/ert375.

Bednarek P. 2010. Chemical warfare or modulators of defence responses – the function of secondary metabolites in plant immunity. Curr. Opin. in Plant Biol. 15, 407–414.

Bednarek P., Pislewska-Bednarek M., Svatos A., Schneider B., Doubsky J., Mansurova M., et al. 2009. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science. 2; 101-106.

Bej S., Basak J. 2014. MicroRNAs: The Potential Biomarkers in Plant Stress Response. Plant. Sci. 748–759.

Bellin D., Asai S., Delledonne M., Yoshioka H. 2013. Nitric oxide as a mediator for defense responses. Mol. Plant Microbe Interact. 26, 271-277.

Bennetzen J.L., Ma J. 2003. The genetic colinearity of rice and other cereals on the basis of genomic sequence analysis. Curr. Opin. Plant Biol. 6, 128-133.

Berrocal-Lobo M., Molina A., Solano R. 2002. Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J. 29, 23-32.

Bigeard J., Colcombet J., Hirt H. 2015. Signaling Mechanisms in Pattern-Triggered Immunity (PTI). Mol. Plant. 8, 521–539.

Boccara M., Sarazin A., Thiébeauld O., Jay F., Voinnet O., Navarro L., Colot V. 2014. The Arabidopsis miR472-RDR6 Silencing Pathway Modulates PAMP- and Effector-Triggered Immunity through the Post-transcriptional Control of Disease Resistance Genes. PLoS Pathogens. 10, 1–16.

Boller T. and Felix G., 2009. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. Ann. Rev. of plant Biol. 60, 379-406.

Boller T. and He S. Y. 2009. Innate Immunity in Plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science. 324, 742–744.

Bolwell G.P., Bindschedler L.V., Blee K.A., Butt V.S., Davies D.R., Gardner S.L., et al. 2002. The apoplastic oxidative burst in response to biotic stress in plants: a tree component system. J. Exp. Bot. 53, 1367–1376.

Bonman J.M., Estrada B.A., Bandong J.M. 1989. Leaf and neck blast resistance in tropical lowlande rice cultivars. Plant disease. 73, 388–390.

Borges F. and Martienssen R.A. 2015. The expanding world of small RNAs in plants. Nat. Rev. Mol. Cell Biol. 16, 727–741.

Borsani O., Zhu J.H., Verslues P.E., Sunkar R., Zhu J.K. 2005. Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in *Arabidopsis*. Cell. 123, 1279-1291.

Bowles D.J. 1990. Defense-Related Proteins in Higher Plants. Ann. Rev. of Biochem. 59, 873-907.

Brodersen P., Sakvarelidze-Achard L., Bruun-Rasmussen M., Dunoyer P., Yamamoto Y.Y., Sieburth L., Voinnet O. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. Sci. 30; 1185-1190.

Bryan G.T., Wu K.S., Farrall L., Hershey H.P., McAdams S.A., Faulk K.N. 2000. A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene Pita. Plant Cell 12, 2033–2046.

Bucciaglia P.A., Smith A.G. 1994. Cloning and characterization of Tag1, a tobacco anther β-1,3-glucanase expressed during tetrad dissolution. Plant Mol. Biol. 24, 903-914.

Buer C.S., Imin N., Djordjevic M.A. 2010. Flavonoids: new roles for old molecules. J. Integr. Plant Biol. 52, 98-111.

Cailliatte R., Lapeyre B., Briat J-F., Mari S., Curie C. 2009. The NRAMP6 metal transporter contributes to cadmium toxicity. Biochemical J. 422, 217–228.

Campo S., Peris-Peris C., Siré C., Moreno A.B., Donaire L., Zytnicki M., et al. 2013. Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the *Nramp6 (Natural resistance-associated macrophage protein 6)* gene involved in pathogen resistance. New Phytologist. 199, 212–227.

Campos-Soriano L., Lupottocand E., San Segundo B. 2013. Investigation of rice blast development in susceptible and resistant rice cultivars using a gfp-expressing *Magnaporthe oryzae* isolate. Plant Pathol. 62, 1030–1037.

Cao H., Li X., Dong X. 1998. Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc. Natl. Acad. Sci. USA. 95, 6531–6536.

Carlucci A., Raimondo M.L., Santos J., Phillips A.J.L. 2012 *Plectosphaerella* species associated with root and collar rots of horticultural crops in southern Italy. Persoonia. 28, 34-48.

Chapman E.J. and Carrington J.C. 2007 Specialization and evolution of endogenous small RNA pathways. Nat. Rev. Genet. 8, 884–896.

Chisholm S.T., Coaker G., Day B., Staskawicz B.J. 2006. Host-microbe interactions: Shaping the evolution of the plant immune response. Cell. 124, 803–814.

Conrath U. 2011. Molecular aspects of defence priming. Trends Plant Sci. 16, 524-531.

Conrath U., Beckers G.J.M., Flors V., García-Agustín P., Jakab G., Mauch F. et al., 2006. Priming: Getting Ready for Battle. MPMI. 19, 1062–1071.

Conrath U., Beckers G.J.M., Langenbach C.J., Jaskiewicz M.R. 2015. Priming for enhanced defense. Ann. Rev Phytopathol. 53, 97-119.

Courtois C., Besson A., Dahan J., Bourque S., Dobrowolska G., Pugin A., Wendehenne D. 2008. Nitric oxide signalling in plants: interplays with Ca2+ and protein kinases. J. Exp. Bot. 59, 155–163.

D. Treutter. 2005. Significance of Flavonoids in Plant Resistance and Enhancement of Their Biosynthesis. Plant Biol. 7, 581–591.

Dangl J.L. and Jones J.D. 2001. Plant pathogens and integrated defence responses to infection. Nature. 411, 826–833.

De Conti Lourenço R.M., da Silva Melo P., Albino de Almeida A.B. 2013. Flavonoids as Antifungal Agents. Antifungal Metabolites from Plants. 283-300. DOI:10.1007/978-3-642-38076-1_10

De Datta S.K. 1981. Principles and practices of rice production. (IRRI, Ed.). Wiley-Interscience.

De Vleesschauwer D., Gheysen G., Höfte M. 2013. Hormone defense networking in rice: tales from a different world. Trends Plant Sci. 18, 555-65.

De Vleesschauwer D., Van Buyten E., Satoh K., Balidion J., Mauleon R., Choi I-R., et al. 2012. Brassinosteroids antagonize gibberellin- and salicylate-mediated root immunity in rice. Plant Physiol. 158, 1833–1846.

De Vleesschauwer D., Xu J., Höfte M. 2014. Making sense of hormone-mediated defense networking: from rice to Arabidopsis. Front. Plant Sci. 5:611.

Dean R., Talbot N.J., Ebbole D.J., Farman M.L., Mitchell T.K., Orbach M.J., Birren, B.W. 2005. The genome sequence of the rice blast fungus *Magnaporthe grisea*. Nature. 434, 980–986.

Dean R., Van Kan J.L., Pretorius Z.A., Hammond-Kosack D., Di Pietro A., Spanu P.D., et al. 2012. The Top 10 fungal pathogens in molecular plant pathology. Mol. Plant Patho. 13, 414–430.

Delaney T.P., 1997. Genetic dissection of acquired resistance to disease. Plant Physiol. 113, 5–12.

Delledonne M., Zeier J., Marocco A., Lamb C. 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. PNAS. 98, 13454-13459.

Denancé N., Ranocha P., Oria N., Barlet X., Rivière M-P., Yadeta K.A., et al. 2013. "*Arabidopsis* wat1 (walls are thin1)-mediated resistance to the bacterial vascular

pathogen, *Ralstonia solanacearum*, is accompanied by cross-regulation of salicylic acid and tryptophan metabolism". Plant J. 73, 225-239.

Diévart A. and Clark S.E. 2004. LRR-containing receptors regulating plant development and defense. Development. 131, 251-261.

Dixon R.A., Achnine L., Kota P., Liu C.J., Reddy M.S.S., Wang L. 2002. The phenylpropanoid pathway and plant defense a genomics perspective. Mol. Plant Pathol. 3, 371-390.

Dogara A.M. and Jumare A.I. 2014. Origin, Distribution and Heading date in Cultivated Rice. Inter. J. of Plant Biol. and Research 2, 2–6.

Domingo C., Andrés F., Tharreau D., Iglesias D.J., Talón M. 2009. Constitutive Expression of *OsGH3.1* Reduces Auxin Content and Enhances Defense Response and Resistance to a Fungal Pathogen in Rice. Mol. Plant-Microbe Interactions. 22, 201–210.

Dong Z., Han M. H., Fedoroff N. 2008. The RNA-binding proteins HYL1 and SE promote accurate in vitro processing of pri-miRNA by DCL1. Proc Natl. Acad. Sci. USA. 105, 9970–9975.

Duan C., Yu J., Bai J., Zhu Z., Wang X. 2014. Induced defense responses in rice plants against small brown planthopper infestation. The Crop Journal. 2, 55–62.

Dufresne M. and Osbourn A.E. 2001. Definition of tissue-specific and general requirements for plant infection in a phytopathogenic fungus. Mol. Plant-Microbe Interact. 14, 300–307.

Durrant W.E., Dong X. 2004. Systemic acquired resistance. Ann. Rev. Phytopathol. 42, 185-209.

Ebrahim S., Usha K., Singh B. 2011. Pathogenesis related (PR) proteins in plant defense mechanism. In: Méndez-Vilas, A. (Ed.) Sci. Against Mic. Patho. 1043–1054.

Ellinger D., Voigt C.A. 2014. Callose biosynthesis in *arabidopsis* with a focus on pathogen response: what we have learned within the last decade. Annals of Bot. doi: 10.1093/aob/mcu120.

Eneydi A.J., Yalpani N., Silverman P., Raskin I. 1992. Localization, conjugation and function of salicyclic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc. Natl. Acad. Sci. USA. 89, 2480-2484.

Falcone-Ferreyra M.L., Rius S.P., Casati P. 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front. Plant Sci. 3:222.

Falcone M.L., Rius S., Casati P. 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front. in Plant Sci. 3:222.

Farmer E.E., Almeras E., Krishnamurthy V. 2003. Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr. Opin. Plant. Biol. 6, 372-378.

Fei Q., Xia R., Meyers B.C. 2013. Phased, secondary, small interfering RNAs in posttranscriptional regulatory networks. Plant Cell. 25, 2400-2415.

Fei Q., Zhang Y., Xia R., Meyers B.C. 2016. Small RNAs Add Zing to the Zig-Zag-Zig Model of Plant Defenses. Mol. Plant Microbe Interact. 29, 165-169.

Franco-Zorrilla J.M., Valli A., Todesco M., Mateos I., Puga M.I., Rubio-Somoza I., et al. 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. Nat Genet. 39, 1033–1037.

Fukuoka S., Saka N., Mizukami Y., Koga H., Yamanouchi U., Yoshioka Y., et al. 2015. Gene pyramiding enhances durable blast disease resistance in rice. Scientific Reports 5: 7773.

Galeotti F., Barile E., Curir P., Dolci M., Lanzotti V. 2008. Flavonoids from carnation (*Dianthus caryophyllus*) and their antifungal activity. Phytochem. 1, 44-48.

Gao F., Wang K., Liu Y., Chen Y., Chen P., Shi Z., et al. 2015. Blocking miR396 increases rice yield by shaping inflorescence architecture. Nature Plants. 2, 1–9.

Garcia-Mata C. and Lamattina L. 2002. Nitric oxide and abscisic acid cross talk in guard cells.Plant Physiol. 128, 790–792.

Garris A.J., Tai T.H., Coburn J., Kresovich S., McCouch S. 2005. Structure and diversity in *Oryza sativa L*. Genetics. 169, 1631-1638.

Gilardi G., Garibaldi A., Gullino M.L. 2013. Seed transmission of *Plectosphaerella cucumerina*, causal agent of leaf spot of *Diplotaxis tenuifolia* in Italy. Phytoparasitica. 41, 411–416.

Glazebrook J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Ann. Rev. Phytopathol. 43, 205-227.

Goff S. 2002. A Draft Sequence of the Rice Genome (*Oryza sativa* L. ssp. japonica). Science 296, 79–92.

Goto M. 1979. Bacterial Foot Rot of Rice Caused by a Strain of *Erwinia chrysanthemi*. Phytopatho. 69, 213-216.

Guan X., Pang M., Nah G., Shi X., Ye W., Stelly D.M., Chen Z.J. 2014. miR828 and miR858 regulate homoeologous MYB2 gene functions in Arabidopsis trichome and cotton fibre development. Nat. Commun. 5:3050.

Gupta O.P., Sharma P., Gupta R.K., Sharma I. 2014. Current status on role of miRNAs during plant-fungus interaction. Physiol Mol. Plant. Pathol. 85, 1–7.

Ham J.H., Melanson R.A., Rush M.C. 2011. *Burkholderia glumae*: next major pathogen of rice?. Mol. Plant Pathol. 12, 329–339.

Hammond-Kosack K.E. and Kanyuka K. 2007. Resistance Genes (R Genes) in Plants. John Wiley & Sons, Ltd. doi: 10.1002/9780470015902.a0020119.

Harper J.F., Binder B.M., Sussman M.R. 1993. Calcium and lipid regulation of an Arabidopsis protein-kinase expressed in *Escherichia coli*. Biochemistry. 32, 3282–3290.

Hendra R., Ahmad S., Sukari A., Shukor Y., Oskoueian E. 2011. Flavonoid Analyses and Antimicrobial Activity of Various Parts of *Phaleria macrocarpa* (Scheff.) Boerl Fruit. Int. J. Mol. Sci. 12, 3422-3431.

Hiei Y., Ohta S., Komari T., Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6, 271–282.

Howard R.J. and Valent B. 1996. Breaking and entering: host penetration by the fungal rice blast pathogen Magnaporthe grisea. Annu. Rev. Microbiol. 50, 491–512.

Huang J., Yang M., Zhang X. 2016. The function of small RNAs in plant biotic stress response. J. Integr. Plant Biol. 58, 312-327.

Huang X., Kurata N., Wei X., Wang Z. X., Wang A., Zhao Q., et al. 2012. A map of rice genome variation reveals the origin of cultivated rice. Nature. 490, 497–501.

Jagadeeswaran G., Saini A., Sunkar R. 2009b. Biotic and abiotic stress down-regulate miR398 expression in *Arabidopsis*. Planta. 229, 1009-1014.

Jagadeeswaran G, Zheng Y, Li YF, et al 2009a. Cloning and characterization of small RNAs from Medicago truncatula reveals four novel legume-specific microRNA families. New Phytol 184, 85–98.

Jakab G., Cottier V., Toquin V., Rigoli G., Zimmerli L., Métraux J.P., et al. 2001. β-aminobutyric acid-induced resistance in plants. Eur. J. Plant Pathol. 107, 29–32.

Jeandet P., Clément C., Courot E., Cordelier S. 2013. Modulation of Phytoalexin Biosynthesis in Engineered Plants for Disease Resistance. Int. J. Mol. Sci. 14, 14136-14170.

Jeandet P., Hébrard C., Deville M-A., Cordelier S., Dorey S., Aziz A., Crouzet J. 2014. Deciphering the Role of Phytoalexins in Plant-Microorganism Interactions and Human Health. Mol. 19, 18033-18056.

Jiang W., Zhou H., Bi H., Fromm M., Yang B., Weeks D.P. 2013. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucl. Acids Res. 41, 1–12.

Jones J.D.G. and Dangl J.L. 2006. The plant immune system. Nature. 444, 323–329.

Jones-Rhoades M.W. 2012. Conservation and divergence in plant microRNAs. Plant Mol. Biol. 80, 3-16.

Jones-Rhoades M.W., Bartel D.P., Bartel B. 2006. MicroRNAs an their regulatory roles in plants. Ann. Rev. of Plant Biol. 57, 19–53.

Kamthan A., Chaudhuri A., Kamthan M., Datta A. 2015. Small RNAs in plants: recent development and application for crop improvement. Front Plant Sci. 06, 1–17.

Kankanala P., Czymmek K., Valent B. 2007. Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. The Plant Cell. 19, 706–724.

Katagiri F., Thilmony R., He S.Y. 2002. The *Arabidopsis Thaliana-Pseudomonas Syringae* Interaction. Arabidopsis Book. 1: e0039.

Katiyar-Agarwal S. and Jin H. 2010. Role of Small RNAs in Host-Microbe Interactions. Ann.Rev. of phytopatho. 48, 225–246.

Katiyar-Agarwal S., Morgan R., Dahlbeck D., Borsani O., Villegas A., Zhu J.K., et al. 2006. A pathogen-inducible endogenous siRNA in plant immunity. Proc. Natl. Acad. Sci. USA. 103, 18002-18007.

Kasschau K.D., Fahlgren N., Chapman E.J., Sullivan C.M., Cumbie J.S., Givan S.A., Carrington J.C. 2007. Genome-wide profiling and analysis of *Arabidopsis* siRNAs. PLoS Biol. 5, 0479–0493.

Kawahara Y., de la Bastide M., Hamilton P.J., Kanamori H., McCombie R.W., Ouyang S., et al. 2013. Improvement of the *Oryza sativa Nipponbare* reference genome using next generation sequence and optical map data. Rice 6:4.

Khang C.H., Berruyer R., Giraldo M.C., Kankanala P., Park S.-Y., Czymmek K., Valent, B. 2010. Translocation of *Magnaporthe oryzae* effectors into rice cells and their subsequent cell-to-cell movement. Plant Cell. 22, 1388–1403.

Kohler A., Schwindling S., Conrath U. 2002. Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the NPR1/NIM1 gene in Arabidopsis. Plant Physiol. 128, 1046-1056.

Kozomara A. and Griffiths-Jones S. 2014. MiRBase: Annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Research. 42, 68–73.

Kuan T., Zhai Y., Ma W. 2016. Small RNAs regulate plant responses to filamentous pathogens. Semin. Cell Dev. Biol. 56,190-200.

Kurihara Y., Takashi Y., Watanabe Y. 2006. The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis. RNA 12, 206–212.

Kurihara Y., Watanabe Y. 2004. Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. Proc. Natl. Acad. Sci. USA. 101, 12753–12758.

Lai Z., Vinod K., Zheng Z., Fan, B., Chen, Z. 2008. Roles of *Arabidopsis* WRKY3 and WRKY4 transcription factors in plant responses to pathogens. BMC Plant Biol. 8:68.

Lakhotia N., Joshi G., Bhardwaj A.R., Katiyar-Agarwal S., Agarwal M., Jagannath A., et al. 2014. Identification and characterization of miRNAome in root, stem, leaf and tuber developmental stages of potato (*Solanum tuberosum L*.) by high-throughput sequencing. BMC Plant Biol. 14, 1–6.

Lee H.J., Park Y.J., Kwak K.J., Kim D., Park J.H., Lim J.Y., et al. 2015. MicroRNA844-guided Downregulation of Cytidinephosphate Diacylglycerol Synthase3 (CDS3) mRNA Affects the Response of Arabidopsis thaliana to Bacteria and Fungi. Mol Plant-Microbe Interact. 28, 892-900.

Legrand S., Valot N., Nicolé F., Moja S., Baudino S., Jullien F., et al. 2010. One-step identification of conserved miRNAs, their targets, potential transcription factors and effector genes of complete secondary metabolism pathways after 454 pyrosequencing of calyx cDNAs from the Labiate *Salvia sclarea L*. Gene. 450, 55–62.

Leubner-Metzger G. 2003. Functions and regulation of beta-1,3-glucanases during seed germination, dormancy release and after-ripening. Seed Sci Res.13, 17–34

Levine A., Tenhaken R., Dixon R., Lamb C. 1994. H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79, 583–593.

Li B., Meng X., Shan L., He P. 2016. Transcriptional Regulation of Pattern-Triggered Immunity in Plants. Cell Host Microbe. 11, 641-650.

Li F., Pignatta D., Bendix C., et al. 2012a. MicroRNA regulation of plant innate immune receptors. Proc. Natl. Acad. Sci. USA. 109, 1790–1795.

Li S., Liu L., Zhuang X., Yu Y., Liu X., Cui X. 2013. MicroRNAs Inhibit the Translation of Target mRNAs on the Endoplasmic Reticulum in Arabidopsis. Cell 153, 562–574.

Li Y., Lu Y.G., Shi Y., Wu L., Xu Y.J., Huang F., et al. 2014. Multiple Rice MicroRNAs Are Involved in Immunity against the Blast Fungus *Magnaporthe oryzae*. Plant Physiol. 164, 1077–1092.

Li Y., Zhang Q., Zhang J., Wu L., Qi Y., Zhou J.M. 2010. Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. Plant Physiol. 152, 2222-2231.

Li Z., Fu B-Y., Gao Y-M., Wang W-S., Xu J-L., Zhang F., et al. 2014. The 3,000 rice genomes project. GigaScience. 3:7.

Lin A., Wang Y., Tang J., Xue P., Li C., Liu L., et al. 2012. Nitric Oxide and Protein S-Nitrosylation Are Integral to Hydrogen Peroxide-Induced Leaf Cell Death in Rice. Plant Physiol. 158, 451–464.

Liu W., Liu J., Triplett L., Leach J.E., Wang G.L., et al. 2014. Novel Insights into Rice Innate Immunity against Bacterial and Fungal Pathogens. Ann. Rev. Phytopathol. 52, 213-241.

Liu X., Williams C.E., Nemacheck J.A., Wang H., Subramanyam S., Zheng C., et al. 2010. Reactive Oxygen Species Are Involved in Plant Defense against a Gall Midge. Plant Physiol. 152, 985–999.

Llave C., Xie Z., Kasschau K.D., Carrington J.C. 2002. Cleavage of *Scarecrow-like* mRNA targets directed by a class of Arabidopsis miRNA. Sci. 297, 2053–2056

Lobbes D., Rallapalli G., Schmidt D.D., Martin C., Clarke J. 2006. SERRATE: a new player on the plant microRNA scene. EMBO Rep. 7, 1052–1058.

Lukasik A., Pietrykowska., Paczek L., Szweykowska-Kulinska Z., Zielenkiewicz P. 2013. High-throughput sequencing identification of novel and conserved miRNAs in the *Brassica oleracea* leaves. BMC Genomics. 14:801.

Luna E., Bruce T.J., Roberts M.R., Flors V., Ton J. 2012. Next-generation systemic acquired resistance. Plant Physiol. 158, 844-853.

Luna E., Pastor V., Robert J., Flors V., Mauch-Mani B., et al. 2011. Callose Deposition: A Multifaceted Plant Defense Response. Mol. Plant-Microbe interact. 24, 183-193.

Machado R.A.R., McClure M., Hervé M.R., Baldwin I.T., Erb M. 2016. Benefits of jasmonate-dependent defenses against vertebrate herbivores in nature. ELife. 5:e13720.

Maclean J., Hardy B., Hettel G. 2013. Rice Almanac (J Maclean, B Hardy, and G Hettel, Eds.). CABI publishing.

Mallory A., Reinhart B., Jones-Rhoades M., Tang G., Zamore P.D., Barton M.K., Bartel D.P. 2004. MicroRNA control of *PHABULOSA* in leaf development: importance of pairing to the microRNA 5' region. EMBO J. 23, 3356-3364.

Martinez-Medina A., Flors V., Heil M., Mauch-Mani B., Pieterse C.M.J., Pozo M.J., et al. 2016. Recognizing Plant Defense Priming. Trends in Plant Sci. 21, 818–822.

Mauch-Mani, B. and Slusarenko, A. J. 1994. Systemic acquired resistance in *Arabidopsis thaliana* induced by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. Mol. Plant-Microbe Interact. 7, 378–390.

Meins F., Neuhaus J.M., Sperisen C., Ryals J. 1992. The primary structure of plant pathogenesis-related glucanohydrolases and their genes, in: T. Boller, F.Jr. Meins, (Eds.), Genes Involved in Plant Defense, Vienna, Springer-Verlag, New York. 245-282.

Mellersh D.G., Foulds I.V, Higgins V.J., Heath M.C. 2002. H2O2 plays different roles in determining penetration failure in three diverse plant-fungal interactions. Science. 29, 257–268.

Meng X., Zhang S. 2013. MAPK cascades in plant disease resistance signaling. Ann. Rev. Phytopathol. 51:, 245-266.

Mew T., Alvarez A., Leach J., Swings J. 1993. Focus on bacterial blight of rice. Plant Dis. 77, 5–12.

Miao J., Guo D., Zhang J., Huang Q., Qin G., Zhang X., et al. 2013. "Targeted mutagenesis in rice using CRISPR-Cas system". Cell Research. 23, 1233-1236.

Mierziak J., Kostyn K., Kulma A. 2014. Flavonoids as Important Molecules of Plant Interactions with the Environment. Molecules. 19, 16240-16265.

Mitchell-Olds T. 2001. *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. Trends in Ecol. and Evol. 16, 693-700.

Miura K., Ikeda M., Matsubara A., Song X., Ito M., Asano K., et al. 2010 OsSPL14 promotes panicle branching and higher grain productivity in rice. Nat. Genet. 42, 545–549.

Morales F.J., Niessen A.I. 1983. Association of spiral filamentous viruslike particles with rice hoja blanca. Phytopathol. 73, 971-974.

Morel J., Fromentin J., Blein J.P., Simon-Plas F., Elmayan T. 2004. Rac regulation of NtrbohD, the oxidase responsible for the oxidative burst in elicited tobacco cell. Plant J. 37, 282–293.

Mouradov A. and Spangenberg G. 2014. Flavonoids: a metabolic network mediating plants adaptation to their real estate. Front. Plant Sci. 5:620.

Naoumkina M.A., Zhao Q., Gallego-Giraldo L., Dai X., Zhao P.X., Dixon R.A. 2010. Genome-wide analysis of phenylpropanoid defence pathways. Mol. Plant Pathol. 11, 829-846.

Navarro L., Dunoyer P., Jay F., Arnold B., Dharmasiri N., Estelle M., et al. 2006. A Plant miRNA contributes to Antibacterial Resistance by Repressing Auxin Signaling. Science. 21, 436–439.

Niu D., Lii Y.E., Chellappan P., Lei L., Peralta K., Jiang C., et al. 2016. miRNA863-3p sequentially targets negative immune regulator ARLPKs and positive regulator SERRATE upon bacterial infection. Nat. Commun. 7:11324.

Nozawa M., Miura S., Nei M. 2012. Origins and evolution of microRNA genes in plant species. Genome Biol. Evol. 4, 230-239.

O'Connell R., Herbert C., Sreenivasaprasad S., Khatib M., Esquerré-Tugayé M.T., Dumas B. 2004. A novel *Arabidopsis-Colletotrichum* pathosystem for the

molecular dissection of plant-fungal interactions. Mol. Plant-Microbe Interact. 17, 272–282.

Onkokesung N., Gális I., von Dahl C.C., Matsuoka K., Saluz H-P., Baldwin I.T. 2010. Jasmonic Acid and Ethylene Modulate Local Responses to Wounding and Simulated Herbivory in *Nicotiana attenuata* Leaves. Plant Physiol. 153, 785-798.

Oliveira M.D.M., Varanda C.M.R., Félix M.R.F. 2016. Induced resistance during the interaction pathogen x plant and the use of resistance inducers. Phytoche. 15, 152–158.

Oñate-Sánchez L., Anderson J.P., Young J. Singh, K.B. 2007. AtERF14, a member of the ERF family of transcription factors, plays a nonredundant role in plant defense. Plant Physiol. 143, 400–409.

Ossowski S., Schwab R., Weigel D. 2008. Gene silencing in plants using artificial microRNAs and other small RNAs. Plant J. 53, 674-690.

Padmanabhan C., Zhang X., Jin H. 2009. Host small RNAs are big contributors to plant innate immunity. Curr. Opin. Plant Biol. 12, 465–472.

Pagnussat G.C., Lanteri M.L., Lombardo M.C., Lamattina L. 2004. Nitric oxide mediates the indole acetic acid induction activation of a mitogen-activated protein kinase cascade involved in adventitious root development. Plant Physiol. 135, 279–286.

Palatnik J.F., Allen E., Wu X., Schommer C., Schwab R., Carrington J.C., Weigel D. 2003. Control of leaf morphogenesis by microRNAs. Nature. 425, 257-263.

Palm M.E., Gams W., Nirenberg H.I. 1995 Plectosporium, a New Genus for Fusarium tabacinum, the Anamorph of Plectosphaerella cucumerina. Mycologia. 87, 397-406.

Pandey S.P. and Somssich I.E. 2009. The Role of WRKY Transcription Factors in Plant Immunity. Plant Physiol. 150, 1648-1655.

Park Y.J., Lee H.J., Kwak K.J., Lee K., Hong S.W., Kang H. 2014. MicroRNA400-guided cleavage of pentatricopeptide repeat protein mRNAs renders *Arabidopsis thaliana* more susceptible to pathogenic bacteria and fungi. Plant and Cell Physiol. 55, 1660–1668.

Peragine A., Yoshikawa M., Wu G., Albrecht H.L., Poethig R.S. 2004. SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in *Arabidopsis*. Genes Dev. 3, 2368–2379.

Pieterse C.M.J., van Wees S.C.M., Hoffl E., van Pelt J.A., van Loon L.C. 1996. SR in Arabidopsis induced by biocontrol bacteria independent of SA accumulation and PRs expression. Plant Cell 8, 1225–1237.

Pieterse C.M.J., Zamioudis C., Berendsen R.L., Weller D.M., Van Wees S.C.M., Bakker P.A.H.M. 2014. Induced Systemic Resistance by Beneficial Microbes. Ann. Rev. Phytopathol. 52, 347–375.

Pitzschke A., Schikora A., Hirt H. 2009. MAPK cascade signalling networks in plant defence. Curr. Opin. Plant Biol. 12, 421-426.

Pré M., Atallah M., Champion A., De Vos M., Pieterse C.M.J., Memelink J. 2008. The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol. 147, 1347–1357.

Pritchard L. and Birch P.R.J. 2014. The zigzag model of plant-microbe interactions: is it time to move on? Mol. Plant Pathol. 15, 865–870.

Pumplin N. and Voinnet O. 2013. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. Nat Rev. Microbiol. 11, 745-760.

Qu S.H., Liu G.F., Zhou B., Bellizzi M., Zeng L., Dai L., et al. 2006. The broad-spectrum blast resistance gene Pi9 encodes a nucleotide-binding site–leucine-rich repeat protein and is a member of a multigene family in rice. Genet. 172, 1901–1914.

Raffaele S. and Rivas S. 2013. Regulate and be regulated: integration of defense and other signals by the AtMYB30 transcription factor. Front. in Plant Sci. 4:98.

Rajagopalan R., Vaucheret H., Trejo J., Bartel D.P. 2006. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Genes Dev. 20, 3407–3425.

Ramos B., González-Melendi P., Sánchez-Vallet A., Sánchez-Rodríguez C., López G., Molina A. 2013. Functional genomics tools to decipher the pathogenicity mechanisms of the necrotrophic fungus *Plectosphaerella cucumerina* in *Arabidopsis thaliana*. Mol. Plant Pathol. 14, 44-57.

Ravaglia D., Espley R.V., Henry-Kirk R.A, Andreotti C., Ziosi V., Hellens R.P. 2013. Transcriptional regulation of flavonoid biosynthesis in nectarine (Prunus persica) by a set of R2R3 MYB transcription factors. BMC Plant Biol. 13:68.

Reguant C., Bordons A., Arola L., Rozès N. 2000. Influence of phenolic compounds on the physiology of Oenococcus oeni from wine. J Appl Microbiol. 88, 1065-1071.

Robert-Seilaniantz A., Grant M., Jones J.D. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Ann. Rev. Phytopathol. 49, 317-343.

Rogers K. and Chen X. 2013. Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell. 25, 2383–2399.

Romeis T., Ludwig A.A., Martin R., Jones J.D.G. 2001. Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J. 20, 5556–5567.

Rondoy M. 2009. Manejo de la pudrición de tallos y vainas de arroz. http://www.oriusbiotech.com/archivos/investigaciones/2011_perumanejo_de_la_pudricin_de_tallos_y_vainas_del_arroz.pdf.

Rubio-Somoza I. and Weigel D. 2011. MicroRNA networks and developmental plasticity in plants. Trends Plant Sci. 16, 258–264.

Ryals J.A., Neuenschwander U.H., Willits M.G., Molina A., Steiner H-Y., Hunt M.D. 1996. Systemic Acquired Resistance. Plant Cell. 8, 1809-1819.

Sanchez P.L., Wing R.A., Brar D.S. 2014. The Wild Relative of Rice: Genomes and Genomics. Plant Genet. and Genomics: 9 Crops and Models 5. Q. Zhang and R.A. Wing (eds.), DOI 10.1007/978-1-4614-7903-1_2, © Springer Science+Business Media New York.

Sánchez-Vallet A., López G., Ramos B., Delgado-Cerezo M., Riviere M.P., Llorente F., et al. 2012. Disruption of abscisic acid signaling constitutively activates *Arabidopsis* resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. Plant Physiol. 160, 2109-24.

Sanchez-Vallet A., Ramos B., Bednarek P., López G., Piślewska-Bednarek M., Schulze-Lefert P., et al. 2010. Tryptophan-derived secondary metabolites in *Arabidopsis thaliana* confer non-host resistance to necrotrophic *Plectosphaerella cucumerina* fungi. Plant journal. 63, 115-127.

Schlaeppi K., Abou-Mansour E., Buchala A., Mauch F. 2010. Disease resistance of Arabidopsis to *Phytophthora brassicae* is established by the sequential actionof indole glucosinolates and camalexin. Plant J. 62, 840–851.

Schuhegger R., Nafisi M., Mansourova M., Petersen B.L., Olsen C.E., Svatoš A., et al. 2006. CYP71B15 (PAD3) Catalyzes the Final Step in Camalexin Biosynthesis. Plant Physiol. 141, 1248-1254.

Seo J.K., Wu J., Lii Y., Li Y., Jin H. 2013. Contribution of small RNA pathway components in plant immunity. Mol. Plant Microbe Interact. 26, 617-625.

Sesma A. and Osbourn A.E. 2004. The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. Nature. 431, 582–586.

Shan T., Rong W., Xu H., Du L., Liu X., Zhang Z. 2016. The wheat R2R3-MYB transcription factor TaRIM1 participates in resistance response against the pathogen *Rhizoctonia cerealis* infection through regulating defense genes. Scientific Reports. 6:28777.

Sharma D., Tiwari M., Pandey A., Bhatia C., Sharma A., Trivedi P.K. 2016. MicroRNA858 Is a Potential Regulator of Phenylpropanoid Pathway and Plant Development. Plant Physiol. 171, 944-959.

Shindo C., Bernasconi G., Hardtke C.S. 2007.Natural Genetic Variation in *Arabidopsis*: Tools, Traits and Prospects for Evolutionary Ecology. Annals of Bot. 99, 1043-1054.

Shivaprasad P.V., Chen H.M., Patel K., Bond D.M., Santos B.A., Baulcombe D.C. 2012. A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. Plant Cell. 24, 859-874.

Shuai P., Liang D., Zhang Z., Yin W. 2013. Identification of drought-responsive and novel *Populus trichocarpa* microRNAs by high-throughput sequencing and their targets using degradome analysis. BMC Genomics. 14:233.

Singha A., Sarmab B.K., Upadhyaya R.S., Singhb H.B. 2013. Compatible rhizosphere microbes mediated alleviation of biotic stress in chickpea through enhanced antioxidant and phenylpropanoid activities. Microbiol. Res. 168, 33–40.

Smita S., Katiyar A., Chinnusamy V., Pandey D.M., Bansal B.C. 2015. Transcriptional Regulatory Network Analysis of MYB Transcription Factor Family Genes in Rice. Front Plant Sci. 6: 1157.

Song G., Jia M., Chen K., Kong X., Khattak B., Xie C., et al., 2016. CRISPR/Cas9: A powerful tool for crop genome editing. The Crop Journal. 4, 75-82.

Soto-Suárez M., Baldrich P., Weigel D., Rubio-Somoza I., San Segundo B. 2017. The Arabidopsis miR396 mediates pathogen-associated molecular pattern-triggered immune responses against fungal pathogens. Scientific Reports. In press.

Spoel S.H., Johnson J.S., Dong X. 2007. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. Proc. Natl. Acad. Sci. USA. 104, 18842–18847.

Spoel S.H., Koornneef A., Claessens S.M.C., Korzelius J.P., Van Pelt J.A., Mueller M.J., et al. 2003. NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell. 15, 760–770.

Staiger D., Korneli C., Lummer M., Navarro L. 2013. Emerging role for RNA-based regulation in plant immunity. New Phytol. 197, 394-404.

Stergiopoulos I., van den Burg H.A., Ökmen B., Beenen H.G., van Liere S., Kema G.H.J., et al. 2010. Tomato Cf resistance proteins mediate recognition of cognate homologous effectors from fungi pathogenic on dicots and monocots. Proc. Natl. Acad. Sci. U.S.A. 107, 7610–7615.

Stotz H.U., Sawada Y., Shimada Y., Hirai M.Y., Sasaki E., Krischke M., et al. 2011. Role of camalexin, indole glucosinolates, and side chain modification of glucosinolate-derived isothiocyanates in defense of Arabidopsis against *Sclerotinia sclerotiorum*. Plant J. 67, 81-93.

Stracke R., Ishihara H., Barsch G.H.A., Mehrtens F., Niehaus K., Weisshaar B. 2007. Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. Plant J. 50, 660-677.

Sunkar R., Zhou X., Zheng Y., Zhang W., Zhu J-K. 2008. Identification of novel and candidate miRNAs in rice by high throughput sequencing. BMC Plant Biol. 8:25.

Sweeney M. and McCouch S. 2007. The complex history of the domestication of rice. Annals of Bot.100, 951–957.

Takahashi R., Ishimaru Y., Senoura T., Shimo H., Ishikawa S., Arao T., et al. 2011. The OsNRAMP1 iron transporter is involved in Cd accumulation in rice. J. of Exp. Botany 62, 4843–4850.

Tanaka K., Choi J., Cao Y., Stacey G. 2014. Extracellular ATP acts as a damage-associated molecular pattern (DAMP) signal in plants. Front. Plant Sci. 5:446.

Thatcher S.R., Burd S., Wright1 C., Lers A., Green P.J. 2015. Differential expression of miRNAs and their target genes in senescing leaves and siliques: insights from deep sequencing of small RNAs and cleaved target RNAs. Plant Cell and Env. 38, 188–200.

Thomma B.P.H.J., Nelissen I., Eggermont K., Broekaert W.F. 1999. Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola.* Plant J. 19, 163-171.

Thomine S., Lelièvre F., Debarbieux E., Schroeder J.I., Barbier-Brygoo H. 2003. AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. Plant J. 34, 685–695.

Ton J., Mauch-Mani B. 2004. Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA dependent priming for callose. Plant J. 38, 119-130.

Torres M.A, Jones J.G.D, Dangl J.L. 2006. Reactive Oxygen Species Signaling in Response to Pathogens. Plant Physiol. 141, 373–378.

Uehara-Ichiki T., Shiba T., Matsukura K., Ueno T.,4 Hirae M., Sasaya T. 2013. Detection and diagnosis of rice-infecting viruses. Front Microbiol. 4:289.

Van Etten H.D., Mansfield J.W., Bailey J.A., Farmer E.E. 1994. Two classes of plant antibiotics: phytoalexins versus 'phytoanticipins'. Plant Cell. 6, 1191-1192.

Van Loon L.C. 1985. Pathogenesis-related proteins. Plant Mol. Biol. 4, 111–116.

Van Loon L.C., Bakker P.A., Pieterse C.M. 1998. Systemic resistance induced by rhizosphere bacteria. Ann. Rev. Phytopathol. 36, 453-483.

Van Loon L.C., Rep M., Pieterse C.M.J. 2006. Significance of inducible defense-related proteins in infected plants. Ann. Rev. of Phytopathol. 44, 135-162.

Van Loon L.C., Van Strien E.A. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. and Mol. Plant Pathol. 55, 85–97.

Van Norman J.M. and Benfey P.N. 2009. *Arabidopsis thaliana* as a Model Organism in Systems Biology. Wiley Interdiscip. Rev. Syst. Biol. Med. 1, 372–379.

Vazquez F. and Hohn T. 2013. Biogenesis and Biological Activity of Secondary siRNAs in Plants. Scientifica. 2013:12.

Vazquez F., Blevins T., Ailhas J., Boller T., Meins J. 2008. Evolution of *Arabidopsis* MIR genes generates novel microRNA classes. Nucleic Acids Res. 36, 6429–6438.

Velazhahan R., Samiyappan R., Vidhyasekaran P. 2000. Purification of an elicitor-inducible antifungal chitinase from suspension-cultured rice cells. Phytoparasitica 28, 131-139.

Vlot A.C., Dempsey D.A., Klessig D.F. 2009. Salicylic Acid, a multifaceted hormone to combat disease. Ann. Rev Phytopathol. 47, 177-206.

Voinnet O. 2009. Origin, Biogenesis, and Activity of Plant MicroRNAs. Cell. 136, 669–687.

Wang F. and Axtell M.J. 2016. AGO4 is specifically required for heterochromatic siRNA accumulation at Pol V-dependent loci in Arabidopsis thaliana. The Plant J. doi: 10.1111/tpj.13463.

Wang L., Du H., Wuyun Ta-na. 2016. Genome-Wide Identification of MicroRNAs and Their Targets in the Leaves and Fruits of Eucommia ulmoides Using High-Throughput Sequencing. Front. Plant Sci. 7:1632.

Wang L., Song X., Gu L., Lia X., Caoa S., Chua C., et al. 2013. NOT2 proteins promote polymerase II-dependent transcription and interact with multiple MicroRNA biogenesis factors in *Arabidopsis*. Plant Cell. 25, 715–727.

Wang M., Yu Y., Haberer G., Marri P.R., Fan C., Goicoechea J.L., et al. 2014. The genome sequence of African rice (*Oryza glaberrima*) and evidence for independent domestication. Nat. Genet. 46, 982–988.

Wang S, Wu K, Yuan Q, Liu X., Liu Z., Lin X., et al. 2012. Control of grain size, shape and quality by OsSPL16 in rice. Nat. Genet. 44, 950–954.

Wang Y., Wang Y., Song Z., Zhang H. 2016. Repression of MYBL2 by Both microRNA858a and HY5 Leads to the Activation of Anthocyanin Biosynthetic Pathway in Arabidopsis. Mol. Plant. 10, 1395-1405.

Wang Z.X., Yano M., Yamanouchi U., Iwamoto M., Monna L., Hayasaka H., et al. 1999. The Pib gene for rice blast resistance belongs to the nucleotide binding and leucine-rich repeat class of plant disease resistance genes. The Plant Journal 19, 55–64.

Watkins J.M., Hechler P.J., Muday G.K. 2014. Ethylene-induced flavonol accumulation in guard cells suppresses reactive oxygen species and moderates stomatal aperture. Plant Physiol. 164, 1707-1717.

Weiberg A., Wang M., Bellinger M., Jin H. 2014. Small RNAs: A New Paradigm in Plant-Microbe Interactions. Ann. Rev. Phytopathol. 52. 495–516.

Williamson B., Tudzynski B., Tudzynski P., van Kan J.A. 2007. Botrytis cinerea: the cause of grey mould disease. Mol. Plant Pathol. 8, 561-580.

Wilson R.A. and Talbot N.J. 2009. Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae*. Nature Rev. Microbiology. 7, 185–195.

Wojtaszek P. 1997. Oxidative burst: an early plant response to pathogen infection. Biochemical J. 322, 681–692.

Wulff E.G., Sorensen J.L., Lübeck .M, Nielsen K.F., Thrane U., Torp J. 2010. *Fusarium spp*. associated with rice Bakanae: Ecology, genetic diversity, pathogenicity and toxigenicity. Environmental Microbiology. 12, 649–657.

Xie Z., Allen E., Wilken A., Carrington J.C. 2005. DICER-LIKE 4 functions in transacting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA. 102, 12984–12989.

Xie Z., Kasschau K.D., Carrington J.C. 2003. Negative feedback regulation of Dicer-Like1 in *Arabidopsis* by microRNA-guided mRNA degradation. Curr. Biol. 13, 784–789.

Yang D.L., Yang Y., He Z. 2013. Roles of plant hormones and their interplay in rice immunity. Mol Plant. 6, 675-85.

Yang L. and Huang H. 2014. Roles of small RNAs in plant disease resistance. J. Integr. Plant Biol. 56, 962–670.

Yang L., Liu Z., Lu F., Dong A., Huang H. 2006. SERRATE is a novel nuclear regulator in primary microRNA processing in *Arabidopsis*. Plant J. 47, 841–850.

Yan Z., Reddy M.S., Ryu C.M., Mc Inroy J.A.,Kloepper J.W. 2002. Induced systemic protection against tomato late blight elicited by PGPR. Phytopathol. 92, 1329–1333.

Ye K.S., Pan S.Q., Kuc J. 1990. Association of pathogenesis-related proteins and activities of peroxidase, β-l,3-glucanase and chitinase with systemic induced resistance to blue mold of tobacco but not to systemic tobacco mosaic virus. Physiol. mol. Plant Pathol. 36, 523–531.

Yellareddygari S.K.R., Reddy M.S., Kloepper J.W., Lawrence K.S., Fadamiro H. 2014. Rice Sheath Blight: A Review of Disease and Pathogen Management Approaches. J. Plant Pathol. Microb. 5:241.

Yu J., Hu S., Wang J., Wong G., Li S., Liu B., et al. 2002. A Draft Sequence of the Rice Genome (*Oryza sativa* L. ssp. indica). Science. 296, 79–93.

Zaidi S., Mansoor S., Ali Z., Tashkandi M, Mahfouz M. 2016. Engineering Plants for Geminivirus Resistance with CRISPR/Cas9 System. 21, 279-281.

Zavaliev R., Ueki S, Epel B.L., Citovsky V. 2011. Biology of callose (β-1,3-glucan) turnover at plasmodesmata. Protoplasma. 1, 117-130.

Zhang B., Pan X., Cannon C.H., Cobb G.P., Anderson T.A. 2006. Conservation and divergence of plant microRNA genes. Plant J. 46, 243-259.

Zhang H., Zhang J., Wei P., Zhang B., Gou F., Feng Z., et al. 2014. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J. 12, 797–807.

Zhang Q., Wing R.A. 2013. Genetics and Genomics of Rice. Springer Science & Business Media.

Zhang X., Xia J., Lii Y. E., Barrera B. E., Zhou X., Gao S., et al. 2012. Genome-wide analysis of plant nat-siRNAs reveals insights into their distribution, biogenesis and function. Genome Biol. 13:3-r20.

Zhang X., Zhao H., Gao S., Wang W.C., Katiyar-Agarwal S., Huang H.D., et al. 2011. Arabidopsis Argonaute 2 regulates innate immunity via miRNA393(∗)-mediated silencing of a Golgi-localized SNARE gene, MEMB12. Mol Cell. 42, 356-366.

Zhang Y., Butelli E., Alseekh S., Tohge T., Rallapalli G., Luo J., et al. 2015. Multi-level engineering facilitates the production of phenylpropanoid compounds in tomato. Nat. Commun. 6:8635.

Zhang Y-C., Yu Y., Wang C-Y., Li Z-Y., Liu Q.,Xu J., et al. 2013. Overexpression of microRNA OsmiR397 improves rice yield by increasing grain size and promoting panicle branching. Nat. Biotechnol. 3, 848–852.

Zhang Z., Liu X., Wang X., Zhou M., Zhou X., Ye X., Wei, X. 2012. An R2R3 MYB transcription factor in wheat, TaPIMP1, mediates host resistance to Bipolaris sorokiniana and drought stresses through regulation of defense- and stress-related genes. New Phytologist. 196, 1155–1170.

Zhao X. and Li L. 2013. Comparative Analysis of MicroRNA Promoters in *Arabidopsis* and Rice. Genomics Proteomics and Bioinformatics. 11, 56–60.

Zhao Y., Zhang C., Liu W., Gao W., Liu C., Song G., et al., 2016. An alternative strategy for targeted gene replacement in plants using a dual-sgRNA/Cas9 design. Scientific Reports. 6: 23890.

Zhengrui Q., Chunlian L., Long M., Liang W. 2014. Novel insights from non-conserved microRNAs in plants. Front. Plant Sci. 5:586.

Zipfel C., 2008. Pattern-recognition receptors in plant innate immunity. Current Opinion in Immunology. 20, 10-16.

ANNEX

Rice transformation

ANNEX. Rice transformation.

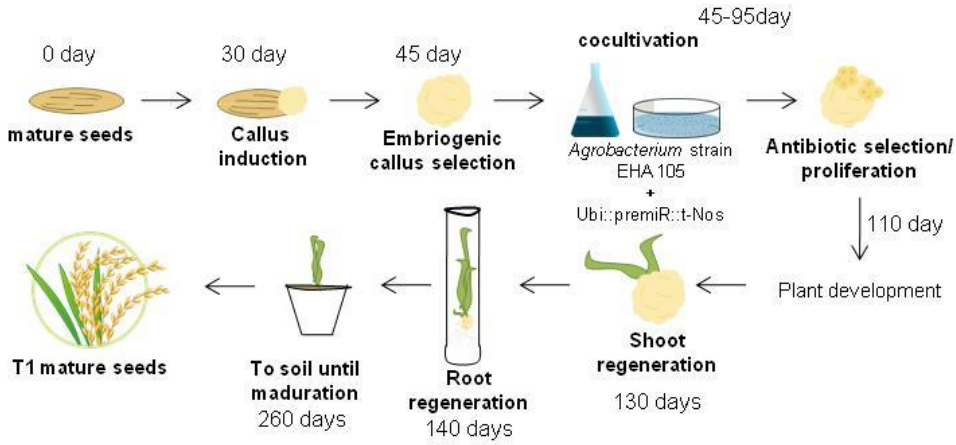
Disinfection of rice seeds

Rice embryogenic calli derived from mature seeds of *Oryza sativa* (*japonica* cv. Nipponbare) were used for stable transformation of rice. De-hulled seeds were surface sterilized in 70% ethanol for 3 min and left in 30% of sodium hypochlorite solution containing Tween®20. The seeds were placed in a rotary shaker for 40 min. After surface sterilization, seeds were rinsed with distilled water several times, including at least 1 h soaking step.

Agrobacterium tumefaciens-mediated transformation of embryogenic calli

Stable transformation of rice embryogenic calli was performed as described by Sallaud et al., (2003). Surface sterilized seeds were placed in NB agar medium and incubated at 28°C in the dark for four weeks. Then, the embryogenic nodular units (0.5-1 mm) from primary callus derived from the scutellum were transferred onto fresh NB agar and incubated for an additional 17 days. Compact embryogenic nodular units (30-50 units; 3 to 5 mm-long) identified by their rough surface, spherical shape and pale yellow colour were selected and used for transformation. Selected embryogenic nodular units were co-cultivated with the *A. tumefaciens* EHA105 carring the binary vector of interest with the presence of acetosyringone. Calli were transferred onto R2S agar selection containing hygromycin (for selection of transformed nodules), and cefataxime and vancomycin (for eliminating *Agrobacterium*), and incubated at 28 °C for 14 days in the dark. During this period, the first transgenic white nodular units appeared, while the rest of the primary calli became necrotic. Calli were transferred and incubated on NBS agar selection at 28°C in the dark. After seven days of incubation, the protuberances arising from primary necrotic calli were separated around the NBS plate using forceps and were incubated at 28°C for 2 weeks in the dark. During these two weeks the putative transgenic calli had evolved into round shape, compact consistency and presented an opaque pale yellow colour. Calli were placed on PRAG agar regeneration (containing hygromycin, cefataxime and vancomycin) and incubated for a further week. The creamy-white with dry appearance calli were transferred to RN agar regeneration, incubated at 28°C for two days in the dark following by an incubation at 28°C for 3-4 weeks using a 12h/12h (day/night) photoperiod. Those calli which acquired green colour and were differentiated into shoots

were transferred to test tubes containing P (rooting medium) agar for 3 weeks to promote tiller and root development before being acclimated in the greenhouse. A scheme of the transformation process is presented below.



Production of transgenic rice by Agrobacterium-mediated transformation of embryogenic calli derived from mature embryos.

Seed production under greenhouse conditions

The primary transgenic and further seed progenies were grown to maturity in the greenhouse. Two different conditions for growth rice plants were used in this work.

1. The substrate used to grow rice plants consisted of 225L of Floratorf substrate (Floragard), 125L of num.3 vermiculite (Europerlita Española), 250g CO_3Ca and 375g of Osmocote (11+11+13+2MgO,Scotts). Once the rice seeds (or seedling) are planted in substrate, the soil surface is kept moist all time (avoid adding large amounts of water to young plants starting tilling). When the rice plants had reached a larger size, the soil is kept well watered at all times adding water to the soil surface using automated dripping. The rice plants were fertilized every two weeks, with a mixture consisting of 100g of Kristalon, 3g of Sequestrene and 3g Hortrilon (diluted 1:50 just before use).
2. Substrate used consisted of 225L of Floratorf substrate (Floragard), 125L of num.3 vermiculite (Europerlita Española), 225g CO_3Ca and 350g of Osmocote (15+9+12+2MgO, Scotts). The rice plants were fertilized every week. In the early stages of development a mixture containing $NH_4H_2PO_4$ 3,10 g/L, K_2SO_4 2,50 g/L, NO_3NH_4 2,56g/L, $(NH_4)_2SO_4$ 21,68g/L, Urea 0,84g/L, EDDHA-Fe 6% 0,62g/L, Micros quelats 0,60g/L diluted 150ml in 10L of water, was used. In the flowering stage the following

solution was used to aid the maturation of the seeds $NH_4H_2PO_4$ 3,70 g/L, K_2SO_4 7,20 g/L, NO_3NH_4 8,04g/L, $(NH_4)_2SO_4$ 10,90g/L, Urea 0,88g/L, EDDHA-Fe 6% 0,62g/L, Micros quelats 0,60g/L diluted 150ml in 10L of water.

The use of the second system has proven to be more convenient compared to the first system, as it helps the plant to develop more vigorously and increasing seed production. Seed production using one or another system to grow different rice genotypes is presented below.

Culture media					
NB		1L	NB selectiu		1L
Macros N6 20x	ml/L	50	Macros N6 20x	ml/L	50
Micros B5 100x	ml/L	10	Micros B5 100x	ml/L	10
Fe-Na EDTA N6/B5 100x	ml/L	10	Fe-Na EDTA N6/B5 100x	ml/L	10
Vitamines B5	ml/L	10	Vitamines B5	ml/L	10
myo-inositol	mg/ml	100	myo-inositol	mg/ml	100
hidrol. Caseïna	mg/ml	300	hidrol. Caseïna	mg/ml	300
Prolina	mg/ml	500	Prolina	mg/ml	500
Glutamina	mg/ml	500	Glutamina	mg/ml	500
2,4-D (10mg/ml)	µl/L	250	2,4-D (10mg/ml)	µl/L	250
Sacarosa	g/L	30	Cefotaxime	µl/L	1.000
Phytagel SIGMA	g/L	2,6	Vancomicina	µl/L	1.000
Ph		5,8	Higromicina	µl/L	1.000
			Sacarosa	g/L	30
R2-líquid		1L	Agarosa tipus I	g/L	7
Macros R2-I 10x	ml/L	100	Ph		6
Macros R2-II 10x	ml/L	100			
Fe-EDTA R2 100x	ml/L	10	PR-AG		1L
Micros R2 100x	ml/L	1	Macros N6 20x	ml/L	50
Vitamines R2	ml/L	1	Micros B5 100x	ml/L	10
2,4-D (10mg/mL)	µl/L	250	Fe-Na EDTA N6/B5 100x	ml/L	10
Glucosa	g/L	10	Vitamines B5	ml/L	10
Acetosiringona	µl/L	1.000	myo-inositol	mg/ml	100
pH		5,2	hidrol. Caseïna	mg/ml	300
			Prolina	mg/ml	500
R2-sòlid		1L	Glutamina	mg/ml	500
Macros R2-I 10x	ml/L	100	BAP (1mg/mL)	µl/L	1.000
Macros R2-II 10x	ml/L	100	ANA (1mg/mL)	µl/L	1.000
Fe-EDTA R2 100x	ml/L	10	ABA (5mg/mL)	µl/L	1.000
Micros R2 100x	ml/L	1	Cefotaxime (400mg/mL)	µl/L	1.000
Vitamines R2	ml/L	1	Vancomicina (100mg/mL)	µl/L	1.000
2,4-D (10mg/mL)	µl/L	250	Higromicina (50mg/mL)	µl/L	1.000
Glucosa	g/L	10	Sacarosa	g/L	30
Acetosiringona	µl/L	1.000	Agarosa tipus I	g/L	7
Agarosa tipus I	g/L	7	Ph		6
pH		5,2			
			RN		1L
R2-sòlid selectiu		1L	Macros N6 20x	ml/L	50
Macros R2-I 10x	ml/L	100	Micros B5 100x	ml/L	10
Macros R2-II 10x	ml/L	100	Fe-Na EDTA N6/B5 100x	ml/L	10
Fe-EDTA R2 100x	ml/L	10	Vitamines B5	ml/L	10
Micros R2 100x	ml/L	1	myo-inositol	mg/ml	100
Vitamines R2	ml/L	1	hidrol. Caseïna	mg/ml	300
2,4-D (10mg/mL)	µl/L	250	Prolina	mg/ml	500
Cefotaxime	µl/L	1.000	Glutamina	mg/ml	500
Vancomicina	µl/L	1.000	BAP (1mg/mL)	µl/L	1000
Higromicina	µl/L	1.000	ANA (1mg/mL)	µl/L	1000
Sacarosa	g/L	30	Sacarosa	g/L	30
Agarosa tipus I	g/L	7	Phytagel	g/L	3,5
pH		6	pH		5,8
P (arrelament)		1L			
Macros-micros-vit MS	g/L	4,4			
Sacarosa	g/L	50			
Phytagel	g/L	2,6			
pH		5,8			

References

Sallaud C., Meynard D., van Boxtel J., Gay C., Bès M., Brizard J.P. 2003. Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. Theor. Appl. Genet. 106, 1396-1408.