Universitat Autònoma de Barcelona

Role of plant miRNAs in disease resistance

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PhD thesis

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Dissertation presented by Raquel Salvador Guirao for the degree of Doctor of Biology and Plant Biotechnology by Universitat Autònoma de Barcelona. This work was performed in Centre for Research in Agricultural Genomics.

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more so that we may fear less.”

Marie Curie

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Y sin darme cuenta han pasado 4 años, así que me gustaría dar las gracias a las personas con las que he compartido de una u otra manera este tiempo y que son a la vez, parte de esta tesis.

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Abbreviations

ABA	Abscisic acid
AGO	ARGONAUTE
amiRNA	Artificial miRNA
bHLH	Basic helix-loop-helix
bZIP	Basic-region leucine-zipper
CDPK	Calcium-dependent protein kinase
cv.	cultivar
DAMP	Damage associated molecular pattern
DCL	Dicer-like
dpi	Days post infection
dsRNA	Double-stranded RNA
EIN2	Ethylene insensitive 2
ET	Ethylene
ETI	Effector triggered immunity
EV	Empty vector
FOC	*Fusarium oxysporum* f. sp. *conglutinans*
H_2O_2	Hydrogen peroxide
hc-siRNA	Heterochromatic siRNA
HEN1	HUA ENANCER 1
hpi	Hours post infection
HR	Hypersensitive response
HST	HASTY
JA	Jasmonic acid
LB	Left border
MAPK	Mitogen-activated protein kinase
Mb	Mega base
MET2	Methyltransferase 2
miRNA	MicroRNA
mRNA	Messenger RNA
MV	Methyl viologen
nat-siRNA	Natural repeat associated siRNA
NPR1	Non-expressor of pathogenesis-related 1
O^{2-}	Superoxide ion
OH^-	Hydroxyl radical
PAMP	Pathogen associated molecular pattern
phasi-RNA	Phased siRNA
PR proteins	Pathogen-related proteins

Pre-miRNA	miRNA precursor
Pri-miRNA	Primary miRNA
PRR	Pattern recognition receptor
PTI	PAMP triggered immunity
R proteins	Resistance proteins
RISC	RNA induced silencing complex
ROS	Reactive oxygen specie
RT-PCR	Reverse transcriptase-polymerase chain reaction
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
SA	Salicylic acid
siRNA	Small-interfering RNA
SOD	Superoxide dismutase
SLRT-qPCR	Stem-loop reverse transcriptase-quantitative chain reaction
sRNA	Small RNA
ta-siRNA	Transacting siRNA
WT	Wild-type

Summary

Plants, due to their sessile nature, evolved a strong innate immune system to defend themselves against pathogen infection. The plant defense response is accompanied by extensive transcriptional reprogramming of gene expression. Essential for a proper defense against pathogens is the transcriptional regulation of genes encoding antimicrobial proteins, defense regulatory proteins (e.g. protein kinases and phosphatases, transcription factors,…), and hormone signaling processes. Evidence also support that small RNAs play an important role in post-transcriptional regulation of gene expression in plant immunity.

MicroRNAs (miRNAs) are small non-coding endogenous RNAs that regulate gene expression at the post-transcriptional level. The regulatory mechanism of miRNAs is based on degradation or translational repression of their target transcripts. Whereas the function of miRNAs in the control of plant development processes has been widely studied, there is still a lack of information about the involvement of miRNAs in plant immunity.

This thesis focused on the study of miRNAs in plant innate immunity. This work has been developed in rice (Chapter I and Chapter II) and Arabidopsis (Chapter III), the model systems for studies on functional genomics in monocotyledonous and dicotyledonous species.

Chapter I describes the function of a polycistronic miRNA, miR166k-166h, in the rice response to infection by the pathogenic fungus *Magnaporthe oryzae*. This fungus is responsible of rice blast disease, one of the most devastating diseases worldwide. Results obtained in this study indicated that miR166k-166h functions as a positive regulator in rice immunity against the blast fungus. Two novel target genes were identified for miR166, the *Ethylene Insensitive 2* (*EIN2*) and *ferredoxin-nitrite reductase* genes. The regulatory role of miRNA species encoded by the miR166h-166k polycistron results from the activity of the miR166* species generated from the miR166k-166h precursor, miR166k* and miR166h* (targeting *EIN2* and *ferredoxin nitrite reductase*, respectively).

Chapter II describes the impact of activation of *Dicer-like 1a* (*DCL1a*), a key component in miRNA biogenesis, during the rice response to *M. oryzae* infection. *OsDCL1a* activation confers susceptibility to the rice blast fungus. The mechanisms by which *OsDCL1a* activation renders the rice plant more susceptible to pathogen infection include: weaker induction of defense-related and diterpenoid phytoalexin genes, and misregulation of genes involved in detoxification of reactive oxygen species (ROS). *OsDCL1a* activation is accompanied by important alterations in the rice miRNAome, including both up-regulation and down-regulation of miRNAs.

Chapter III investigates the involvement of miR773 in Arabidopsis immunity. This miRNA guides cleavage of *methyltransferase2* (*MET2*) transcripts. Interference with miR773 by target mimics (*MIM773* plants) increases resistance to infection by necrotrophic and hemibiotrophic fungal pathogens (*Plectosphaerella cucumerina*, *Fusarium oxysporum* f. sp. *conglutinans*, *Colletotrichum higginsianum*), whereas *MIR773* overexpression and *MET2* silencing confers susceptibility to fungal infection. *MIM773* plants exhibit stronger induction of defense responses during pathogen infection.

Altogether, the results obtained during this PhD thesis demonstrate that miRNAs and DCL1, a component of the miRNA biogenesis pathway, mediate innate immune responses in rice and Arabidopsis. A better knowledge of miRNA function will be useful in designing novel strategies for crop protection.

Resumen

Las plantas, debido a su naturaleza sésil, han desarrollado un potente sistema inmune para defenderse de la infección por patógenos. La respuesta de defensa va acompañada de una extensiva reprogramación de la expresión de genes codificantes para proteínas antimicrobianas, factores de transcripción, proteínas de señalización y proteínas de regulación hormonal relacionadas con la defensa. Se ha demostrado que los pequeños RNAs juegan un papel importante en la regulación post-transcripcional de la expresión génica en la inmunidad de plantas

Los microRNAs (miRNAs) son pequeños RNA endógenos no codificantes, reguladores clave de la expresión génica a nivel post-transcripcional. Sus mecanismos reguladores están basados en la degradación o en la represión de la traducción de sus tránscritos diana. La función reguladora de los miRNAs en el desarrollo de plantas ha sido ampliamente estudiada, sin embargo, la información sobre el papel de los miRNAs en la respuesta de defensa frente a patógenos está más limitada.

Esta tesis comprende el estudio de miRNAs en la inmunidad innata de las plantas. El trabajo ha sido desarrollado en arroz (Capítulo I y Capítulo II) y Arabidopsis (Capítulo III). Estas plantas sirven como modelo para estudios de genómica funcional en plantas monocotiledóneas y dicotiledóneas, respectivamente.

El **Capítulo I** describe la función de un miRNA policistrónico, miR166k-166h, en la respuesta del arroz a la infección con el hongo patógeno *Magnaporthe oryzae*. Este hongo es el responsable de la piriculariosis, una de las enfermedades más devastadoras del arroz a nivel mundial. Los resultados obtenidos en este estudio indican que miR166k-166h funciona como regulador positivo de la respuesta inmune conta *M.oryzae*. Dos nuevos genes diana han sido identificados para el miR166, *"Ethylene insensitive 2"* (*EIN2*) y *"ferredoxin-nitrite reductase"*. El papel regulador de las especies contenidas en el policistrón miR166k-166h resulta de la actividad de las especies miR166*: miR166k* y miR166h* (cortando *EIN2* y *ferredoxin-nitrite reductase* respectivamente).

El **Capítulo II** describe el impacto de la activación de *Dicer-like 1a* (*DCL1a*), un componente clave en la biogénesis de miRNAs, durante la respuesta de defensa frente a *M. oryzae*. La activación de *DCL1a* desencadena susceptibilidad a este patógeno. Los mecanismos por los cuales la activación de *DCL1a* produce susceptibilidad son diversos, desde la represión de la expresión de genes de defensa y de genes implicados en la síntesis de fitoalexinas diterpenoides, hasta la producción de especies reactivas de oxígeno (ROS). La activación de *DCL1a* está además acompañada de una importante alteración del miRNAoma de arroz, incluyendo acumulación y represión de miRNAs.

El **Capítulo III** investiga el papel del miR773 de Arabidopsis en la inmunidad innata. Este miRNA promueve el corte de tránscritos del gen *methyltransferase2* (*MET2*). Cuando la acción del miR773 se ve reprimida por el mecanismo "*target mimic*" de imitación de diana, las plantas (*MIM773*) son más resistentes a la infección fúngica pon hongos necrótrofos y hemibiótrofos (*Plectosphaerella cucumerina*, *Fusarium oxysporum* f. sp. *conglutinans* and *Colletotrichum higginsianum*). Sin embargo, tanto la sobreexpresión del miR773 como la mutación de *met2* confieren susceptibilidad a la infección fúngica.

En conjunto, los resultados obtenidos en esta tesis doctoral demuestran que los miRNAs y los genes de biogénesis de miRNAs son componentes importantes de las respuestas inmunes frente patógenos, tanto en arroz como en Arabidopsis. Un mejor conocimiento de la función de miRNAs puede ser de utilidad para el diseño de nuevas estrategias para la protección de cultivos.

General Introduction

General introduction

1. Rice

1.1 Rice cultivation: origin and diversity

Rice is a member of *Poaceae* family of monocotyledonous flowering plants (grasses). It is an herbaceous plant with a fasciculate root system and erect, cylindrical stems. The rice leaves are lanceolated, with parallel enervation, and are attached to the stem through the sheath in an alternate distribution (**Figure 1 A**). Tillers produce panicle inflorescences that consist of a main axis divided into secondary branches (**Figure 1 B**). The secondary branches carry the spikelet (**Figure 1 C**). The rice flower is hermaphroditic and contains six stamens and a pistil with feathery stigma (**Figure 1 D**). The grain of rice is a caryopsis type fruit and the embryo is at the ventral side of the grain. The remaining part of the grain is occupied by the starchy endosperm (**Figure 1 E-F**).

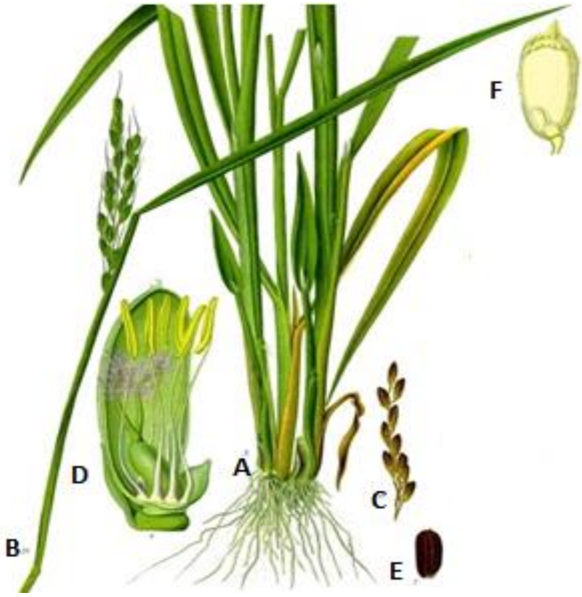

Figure 1. Rice morphology. A. Roots and stems of a rice plant. B. Panicle. C. Spikelet. D. Flower. E. Rice grain. F. Scheme of a rice seed. Images modified from Franz Eugen Köhler, Köhler's Medizinal-Pflanzen, 1897.

The genus *Oryza* comprises 2 cultivated and 22 wild species representing different genome types, both diploid and tetraploid (AA, BB, CC, EE, FF, GG, BBCC, CCDD, KKLL, and HHJJ) genomes (Sanchez et al. 2013). Wild species include *O. officinalis, O. punctata, O. rhizomatis, O. rufipogon, O. nivara, O. meridionalis, O. granulate,* among others. The cultivated species (AA genome) are *Oryza sativa* (the Asian rice) and *Oryza glaberrima* (the African rice). Within *O. sativa* group we can find two main groups: *O. sativa spp. Japonica* and *O. sativa spp. Indica.* The *O. sativa japonica* group is further differentiated into *temperate japonica* (*japonica*) and *tropical japonica* (*javanica*) varieties (Sweeney and McCouch 2007). *O. sativa* domestication took place approximately 9.000 years ago from *O. rufipogon* (perennial) and *Oryza nivara* (annual) in the area surrounded by eastern China to eastern India, including both of these current countries in addition to Vietnam, Laos and Thailand (Kovach et al. 2007; Dogara and Jumare 2014). *O. sativa spp japonica* was first domesticated around the middle region of Pearl river in Southern China and *O. sativa spp indica* was subsequently developed from crosses between japonica and local wild rice as the initial cultivars spread into southern Asia (Huang et al. 2012).

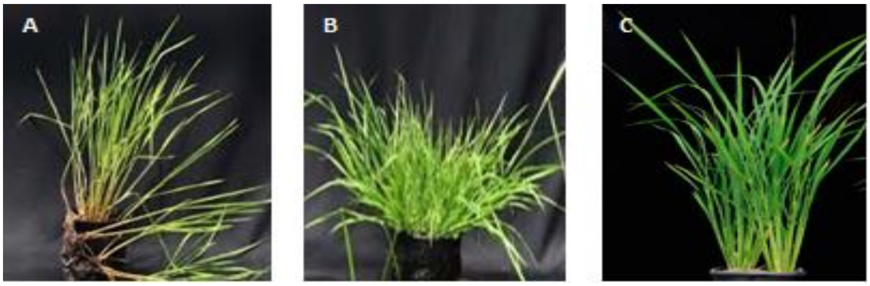

Figure 2. Domestication from *O. rufipogon* and *O. nivara* to *O. sativa*. A. Aerial part of *O. rufipogon*. **B.** *O. nivara* **C.** *O. sativa*.

More recently than *O. sativa*, *O. glaberrima* domestication occurred from *O. barthii* in Niger river's Delta, in the occidental Africa (Wang et al. 2014).

Figure 3. Domestication from *O. barthii* to *O. glaberrima*. A. Panicles of *O. barthii*. **B.** Panicles of *O. glaberrima*. **C.** Seeds of *O. barthii* and *O. glaberrima*.

Oryza sativa has a diploid genome (2n=24). It has the smallest genome of the major cereal genomes (430 Mb) and contains around 40.000 coding genes (Goff et al. 2002). For this reason, rice is considered as a model specie in cereal genomics.

The genome sequence of *O. sativa* (*O. sativa spp. Japonica* cv. Nipponbare; *O. sativa spp. Indica*) is available (Goff et al. 2002; Yu et al. 2002; Kawahara et al. 2013). Moreover, the *3000 Rice Genomes Project* has made available the genome sequence of 3024 rice varieties from 89 countries (The 3000 rice genomes project 2014) thanks to the effort of the Chinese Academy of Agricultural Sciences, the International Rice Research Institute and the Beijing Genomics Institute. The African rice *O. glaberrima* sequence is also available (Wang et al. 2014) as well as the sequences of 1504 mutants in the short-cycle rice variety Kitaake (*O. sativa spp* japonica) (Li et al. 2017a). Moreover, efficient rice transformation protocols have been developed and public cDNA libraries and mutants collections are available. Some examples of mutant rice collections are: the *Taiwan Rice Insertional Mutants* (TRIM; http://trim.sinica.edu.tw/), *Oryza Tag-Line* (http://oryzatagline.cirad.fr/), *Rice Mutant Database* (RMD; http://rmd.ncpgr.cn/), *POSTECH-Rice Insertion Databse* (http://cbi.khu.ac.kr) and *Tos17 Insertion mutant database* (http://tos.nias.affrc.go.jp/).

1.2 Rice production

Rice is the second most important cereal in the world after maize and the primary cereal for human consumption. Thus, rice is the main staple food for half of the world’s population, contributing to 80% of the caloric intake in Asian countries and more than 23% for global world population (Dawe et al. 2010) (**Figure 4**).

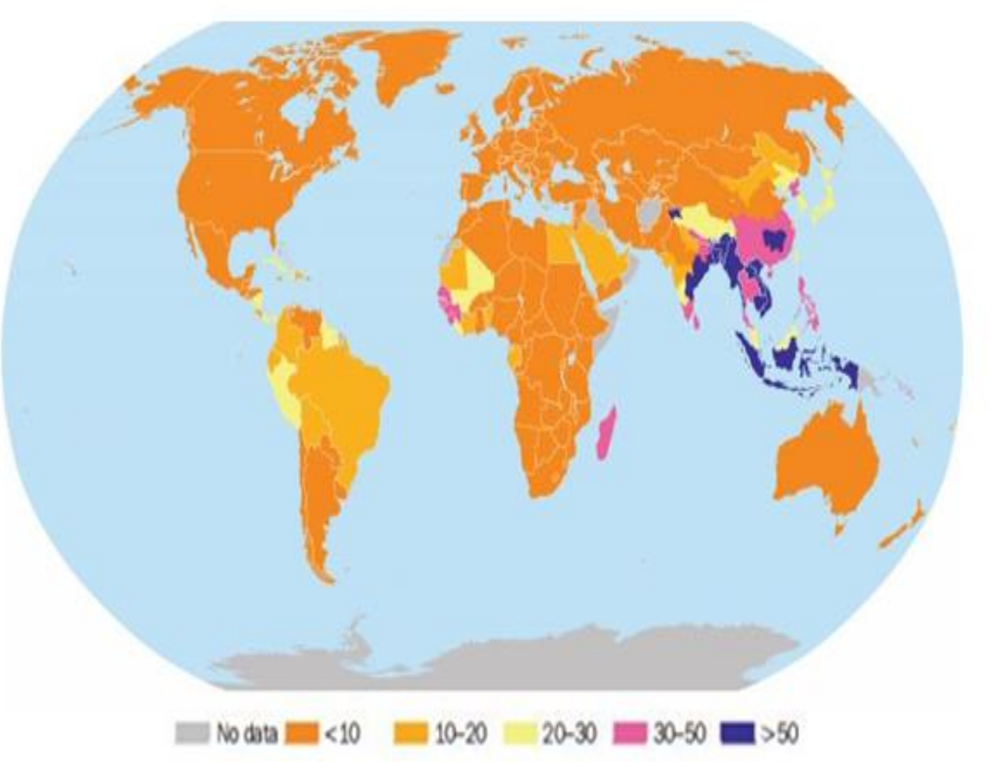

Figure 4. Percentage of calories coming from rice over the world. Taken from Dawe et al. 2010.

In Europe, Italy is the mayor rice producer with over a half of the total rice production, followed by Spain (approximately 30% of the total rice produced), France and Greece (FAOSTAT 2016). Rice can be cultivated in different ecosystems, from temperate areas to tropical ones, and from sea level to geographical areas up to 2000 m altitudes (**Figure 5**).

Figure 5. Rice fields. A. Rice terraces. **B.** Cultivation in irrigation plots. **C.** Cultivation in paddy plots. **D.** Cultivation in drought soils (experimental station).

1.3 Rice diseases

Diseases caused by insect pests and pathogens are responsible of important cereal losses, including rice losses. The major rice bacterial pathogens are *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight, and *X. oryzae* pv. *oryzicola,* responsible of the bacterial leaf streak (Swings et al. 1990; Niño-Liu et al. 2006). Other rice bacterial pathogens are *Erwinia chrysanthemi* (foot rot) (Goto 1979), *Burkholderia glumae* (grain rot) and *Pseudomonas fuscovaginae* (sheath brown rot)(Cottyn et al. 1996).

Regarding fungal pathogens, one of the most devastating pathogenic fungus is *Magnaporthe oryzae,* the causal agent of the rice blast disease (Wilson and Talbot 2009). Other fungal pathogens of importance in rice fields are *Rhizoctonia solani*, causing sheath blight, (Yellareddygari et al. 2014) and *Gibberella fujikuroi* causing bakanae, also known as foolish seedling disease (Wulff et al. 2010).

Contrary to bacterial and fungal diseases which are widespread in different geographical areas, the incidence of viral diseases depends on the geographical area. The *Rice Hoja Blanca Virus* (RHBV) is found in central and South America (Morales and Niessen 1983), *Rice Yellow Mottle Virus* (RYMV) in Africa (Kouassi et al. 2005) and *Rice Stripe Virus* (RSV) in Asia, among others (Ling 1972).

1.3.1 Magnaporthe oryzae

Magnaporthe oryzae, a new species separated from *M. grisea* (Couch and Kohn 2002), is a filamentous ascomycete fungus with a hemibiotrophic life style (Wilson and Talbot 2009). Annual losses in rice production caused by the rice blast fungus were estimated to feed 60 million people for one year (Zeigler et al. 1994; Nalley et al. 2016). On the other hand, the rice production needs to be increased more than 40% by 2030 to meet the people's demand (Khush 2005).

In leaves, the life cycle of *M. oryzae* starts when a three-celled conidium attaches to the leaf cuticle (**Figure 6 A**). Then, a single polarized germ tube emerges from the spore and grows along the leaf surface (**Figure 6 B**). The germ tube differentiates at its tip into an appressorium **(Figure 6 C**). Once formed, the appressorium maturates and accumulates a high concentration of solutes in order to generate turgor, which is translated into mechanical force to penetrate into leaf cuticle through a specialized hyphae, the penetration peg (Howard and Valent 1996; Wilson and Talbot 2009) (**Figure 6 D**). Hyphae grow and ramify in the mesophyll and finally their morphology change from thin hyphae (typical from the biotrophic stage) to thick and bulbous hyphae (necrotrophic stage). The fungus invades adjacent cells by the plasmodesmata. At approximately four days after the infection, the blast disease lesions, elliptical or spindle-shaped, with dark borders, develop on the infected leaves (Khang et al. 2010; Campos-Soriano et al. 2013). The fungus sporulates from the lesions, allowing the disease to spread rapidly to adjacent rice plants (**Figure 6 E-F**). Under laboratory conditions, *M. oryzae* is able to infect roots (Sesma and Osbourn 2004; Campos-Soriano et al. 2013).

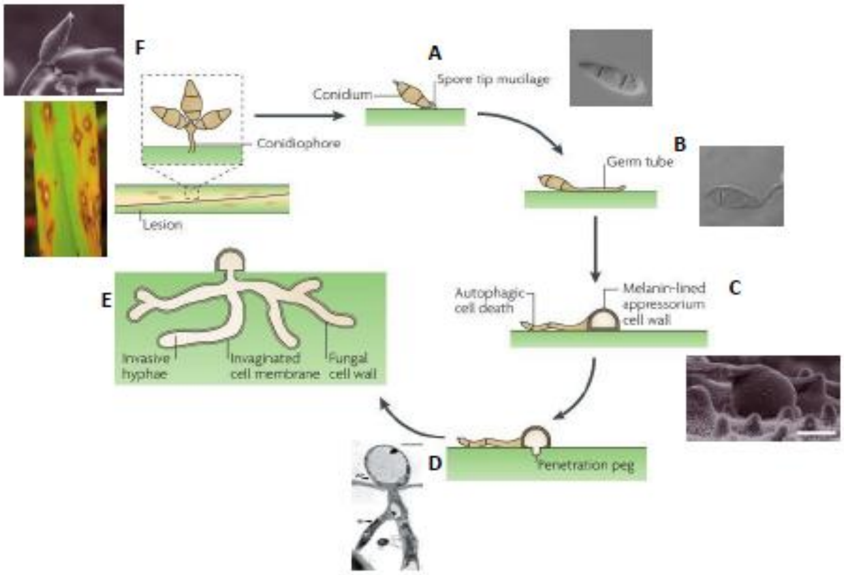

Figure 6. Life cycle of *Magnaporthe oryzae*. Adapted from Wilson and Talbot 2009.

An entire life cycle can be completed in one week. The optimal temperature for blast development is 25-28ºC and high humidity conditions.

Traditionally, blast disease control has been carried out using chemical fungicides, but they are expensive and can be toxic for environment. Moreover, the use of pesticides has proven not to be effective for the control of this fungus due to the continuous appearance of new fungal races. In recent years, plant breeding has achieved significant progress towards the enhancement of blast resistance with the identification of a broad array of blast resistance (R) genes. More than 85 dominant R genes and approximately 350 QTLs to resistance to rice blast have been identified and some of them have been molecularly characterized (Ballini et al. 2008; Yan et al. 2017). Some examples of blast R genes are: Pib (Wang et al. 1999), Pita (Bryan et al. 2000), Pi9 (Qu et al. 2006), Pi2/Pizt (Zhou et al. 2006), Pid2 (Chen et al. 2006), Pi36 (Liu et al. 2007b), Pi37 (Lin et al. 2007a), Pik-m (Ashikawa et al. 2008), Pi5 (Lee et al. 2009), Pit (Hayashi and Yoshida 2009), Pid3 (Shang et al. 2009) and Pi21 (Fukuoka et al. 2009). Several of these blast resistance genes have demonstrated their ability to confer resistance to various blast pathotypes, and are being effectively used in breeding programs to increase blast resistance in rice. However, none of the blast resistance genes so far identified appears to confer durable resistance against *M. oryzae* and R-mediated resistance is not effective against all *M. oryzae* isolates. Because of the genetic variability of *M. oryzae* and race changes in blast populations, resistant cultivars with single-gene resistance have been shown to lose effectiveness after a few years (Lee et al., 2009). Durable resistance in breeding programs requires gene pyramiding strategies for the simultaneous expression of more than one R gene in the same cultivar.

2. Arabidopsis

Arabidopsis belongs to the *Brassicaceae* family, which includes cultivated species such as cabbage, broccoli and radish. The leaves of Arabidopsis plants form a rosette at the base of the plants with a few leaves also in the stem (caulinar leaves) (**Figure 7 A-B)**. Arabidopsis produces hermaphrodite flowers (**Figure 7 C)**. The fruit resulting from the fecundation is a cylindrical and elongated silique that contains approximately 30 seeds in a row.

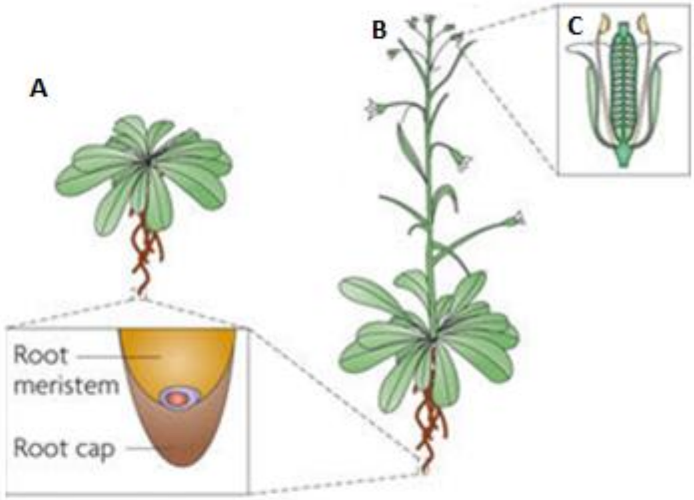

Figure 7. Morphology of Arabidopsis plants. A. Rosette formation in adult plants. B. Rosette, stem and flowers in a reproductive stage plant. C. Flower of Arabidopsis. Adapted from Kaufmann et al. 2010.

Arabidopsis thaliana (2n=10) was the first plant species with a sequenced genome (The Arabidopsis Genome Initiative 2000). It has a small genome of approximately 135 Mb distributed in 5 chromosomes. In addition to its short life cycle (around 6 weeks) Arabidopsis has a prolific seed production. Effective Agrobacterium-mediated transformation procedures have been developed for Arabidopsis (Clough and Bent 1998). Upto 1135 Arabidopsis accessions (http://1001genomes.org) have been collected around the world and are available at different seed stocks, being the Arabidopsis Biological Resource Center one of the most important. All these characteristics and the huge availability of mutant lines led plants scientists to adopt Arabidopsis as a model organism for molecular genetic research.

Many pathosystems have been established in *Arabidopsis thaliana*. Some pathogens, for instance *Pseudomonas syringae* and *P. viridiflava,* resulted to be natural (Jakob et al. 2002), but pathogens from closely related species have been also used in Arabidopsis for the study of plant defense mechanisms. After *Pseudomonas syringae,* the interaction with the bacterial pathogen *Ralstonia solanacearum* has been also extensively studied (Deslandes et al. 2002; Van der Linden et al. 2013).

Fungal pathogens in Arabidopsis include obligate biotrophs (*Puccinia sp.*), hemibiotrophs (*Phytophtora sp., Colletotrichum sp)* and necrotrophs (*Botrytis sp., Fusarium sp.* and *Plectosphaerella sp*).

2.1 *Plectosphaerella cucumerina*

Plectosphaerella cucumerina (anamorph: *Plectosporum tabacinum*), previously named as *Fusarium tabacinum,* is a filamentous ascomycete able to survive saprophytically in soil (Palm 1995). This fungus has a necrotrophic life style and causes sudden death and blight disease in a variety of crops, for instance melon, tomato and pepper (Carlucci et al. 2012). In addition, *P. cucumerina* is able to infect Arabidopsis (Ramos et al. 2013) reason why this fungus emerged as a popular pathogen to study plant defense reactions to necrotrophic fungi in *Arabidopsis thaliana* (Ton and Mauch-Mani 2004; Gamir et al. 2014).

3. Plant Innate Immunity

In nature, plants are exposed to a variety of potential pathogenic microorganisms. To protect themselves against pathogens, plants have an innate immune system (Jones and Dangl 2006). The first mechanism of plant defense is characterized by a basal resistance response, called pattern-triggered immunity (PTI), which prevents non-adapted microbes from infecting plants. The surface pattern recognition receptors (PRRs) act as initial radars to recognize pathogen-associated molecular patterns (PAMPs). PAMPs can be proteins, carbohydrates, lipopolysaccharides and other molecules essential for the microbes. Recognition by PRRs induces a rapid auto and trans-phosphorylation of the receptors (Macho and Zipfel 2014). One of the earliest physiological responses to PAMP recognition is callose deposition at the cell wall, as well as the influx of extracellular Ca^{2+} into the cytosol. Ion transportation through the plasma membrane leads to an extracellular alkalinization and depolarization of the plasma membrane (H^+ and Ca^{2+} input, K^+ and Cl^- output), and activation of protein phosphorylation/dephosphorylation processes in which calcium-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs) are involved (Jeworutzki et al. 2010) (**Figure 8**). Reactive oxygen species (ROS) also play a central role in plant defense against various pathogens (Brien et al. 2012). Thus, perception of PAMPs induces an extracellular oxidative burst, which requires the function of NADPH oxidase for production of membrane-impermeable O^{2-} in the apoplast, which is then converted to H_2O_2 by the activity of peroxidases (**Figure 8**). H_2O_2 is membrane-permeable and can enter into the cytosol and organelles. H_2O_2 functions as a signalling molecule for the activation of defense responses as well as an antimicrobial agent. H_2O_2 also serves for cell wall lignification (barrier for pathogen penetration). However, the excess of ROS molecules can be toxic for plant cells, due to peroxidation of lipids or proteins and DNA damage. For that reason, plants have mechanisms to regulate the ROS through enzymatic systems (peroxidases, catalases, superoxide dismutases or glutathione S-transferases) or non-enzymatic systems (production of antioxidant compounds like glutathione, flavonoids or carotenoids). In addition to ROS, the production of nitric oxide (NO) is also a rapid response to pathogen infection. Nitric oxide participates in cell death programs and stimulates signal transduction pathways through protein kinase and hormone signalling pathways (Courtois et al. 2008). Finally, PTI is characterized by the accumulation of pathogenesis-related proteins (PRs), some of them exhibiting antimicrobial activity (Van Loon 1999) (**Figure 8**). Among them are chitinases, defensins and thionins (Mehdy 1994). In addition to PAMPs, plants can activate PTI by the recognition of damage-associated molecular patterns (DAMPs). DAMPs are molecules passively released from dying cells due to damage or infection-induced necrosis (Choi and Klessig 2016). An example of DAMP are oligogalacturonides (Ferrari et al. 2013).

Pathogens display a remarkable propensity for adapting to particular hosts, and produce virulence factors known as effectors, which interfere with PTI nullifying basal defense. In turn, plants have evolved another layer of immunity based on the direct or indirect recognition of microbial effectors by host receptors, the so called Resistance (R) proteins (**Figure 8**). Resistance (R) proteins are nucleotide-binding (NB)/leucine-rich-repeat (LRR)(NB-LRR) receptors. This response is called effector-triggered immunity (ETI) and it is in charge of reinstate and amplify PTI basal transcriptional programs and antimicrobial defenses. ETI is host and pathogen-specific (Cui et al. 2015).

ETI is frequently associated to an hypersensitive response (HR) in which a controlled cell death is produce around the infection point, delimiting the infection propagation (Jones and Dangl 2006). Resistance (R) genes are the most rapidly evolving of plant genes and their expansion in number and sequence diversification has been driven by pathogen pressure for new receptor recognition capabilities (Jacob et al. 2013). Depending of their N-terminal domain R proteins can be divided in two subclasses: the TIR-NB-LRR, also called TIR-NBS-LRR, that contain the Toll-interleukin 1(TIR) receptor domain and are found only in dicots, and the CC-NB-LRR or CC-NBS-LRR, that have a coiled coil (CC) domain and are present in both dicots and monocots. Other types of R proteins are characterized by the presence of an unique LRR extracellular domain (Stergiopoulos et al. 2010). In rice, resistance to bacterial and fungal pathogens is conferred by both resistance genes (ETI) and basal resistance (PTI) (Liu et al. 2014). A broad array of blast resistance R genes has been described. Some examples are *Pita-2, Pi39(t), Pi20(t), Pi5(t), Pi15, PiCO39(t),* among others. Most of these *R* genes have been included in breeding programs and provide resistance to specific pathotypes. Due to the pathogenic variability, resistance in these new rice varieties is rapidly broken down (Tanweer et al. 2015).

Regarding transcription factors, two large families of transcription factors are known to be involved in the rice defense response to *M. oryzae*. They include transcription factors belonging to the *NAC* family (*NAC6*, *NAC19, NAC122* and *NAC131* are involved in rice blast resistance) (Lin et al. 2007b; Nuruzzaman et al. 2010; Nakashima et al. 2007; Sun et al. 2013) and the *WRKY* family (*WRKY1, WRKY13, WRKY22, WRKY31, WRKY45, WRKY53, WRKY82* and *WRKY89* have shown to play important roles in resistance to *M. oryzae*) (Zhang et al. 2008; Qiu and Yu 2009; Wang et al. 2011a; Abbruscato et al. 2012; Cheng et al. 2015).

Phytohormones play a crucial role in disease resistance. The main hormones playing a role in plant defense are: salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA). These hormones are able to modulate plant resistance by metabolomic pathways or stimulating the expression of defense-related genes, such as *PR* genes (Denancé et al. 2013). Historically, salicylic acid has been associated to biotrophic pathogens, and jasmonic and ethylene to necrotrophic pathogens. Antagonistic or synergic interactions between these defense-related hormones during plant infection have been demonstrated (Glazebrook 2005; Tamaoki et al. 2013). Secondary metabolites as phytoalexins are also essential for plant defense, as described below(Hasegawa et al. 2010; Yamane 2013).

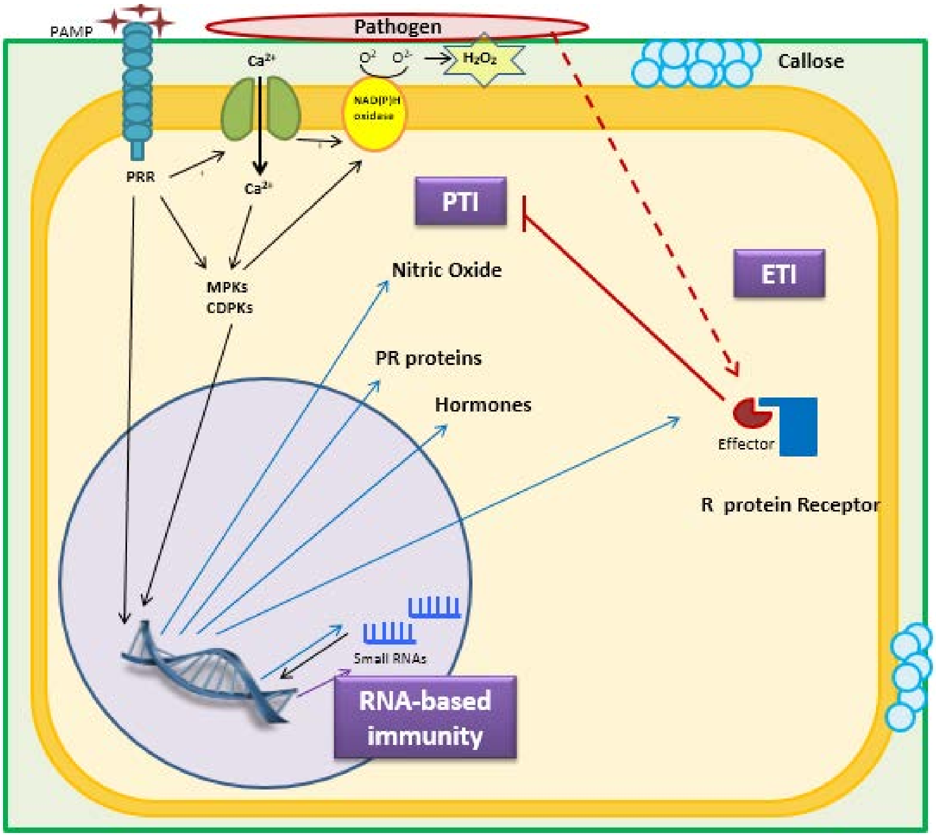

Figure 8. Defense mechanism against fungal and bacterial pathogens in plants. Typical responses to basal (PTI) and pathogen-specific (ETI) resistance, as well as the RNA-based immune system are represented. PAMPS, pathogen activated molecular pattern; PRR, pattern recognition receptor; MPKs, mitogen-activated protein kinases; CDPKs, calcium-dependent protein kinases.

3.1 Phytoalexins in plant immunity

Phytoalexins are antimicrobial compounds, with a low molecular weight, that are both synthesized by and accumulated in plants after exposure to microorganisms (Paxton 1980; Hasegawa et al. 2010; Yamane 2014). They are important players in the plant defense system against a variety of pathogens. A variety of phytoalexins are produced in many dicot and monocot species, not only in response to pathogen infection, but also in response to elicitor treatment (Tsuji et al. 1992; Ahuja et al. 2012). Different reports indicated that phytoalexins accumulate rapidly at the site of attempted infection in sufficient quantities to inhibit the growth of fungi (Sato et al. 1971; Yoshikawa et al. 1978) and bacteria (Keen and Kennedy 1974). Structures of phytoalexins were found to be diverse, including flavonoid, isoflavonoid, diterpenoid, sesquiterpenoid and indole (Grayer and Kokubun 2001). In rice, different types of phytoalexins have been isolated and characterized, including one flavonoid, sakuranetin, and several diterpenoid phytoalexins. The diterpene type of phytoalexins, the major phytoalexins in rice, contain four structurally distinct types: momilactones A and B, oryzalexin S, oryzalexins A to F and phytocassenes A through E, and their biosynthetic genes are identified (Kanno et al. 2006; Xu et al. 2007). Some phytoalexin biosynthesis genes form clusters in chromosomes 2 and 4 (Shimura et al. 2007; Yamane 2013), and these clusters are under the control of distinct transcription factors, for instance *OsTGAP1* (Okada et al. 2009), *OsbZIP79* (Miyamoto et al. 2015), *OsbHLH025* (Yamamura et al. 2015) and *OsWRKY45* (Akagi et al. 2014).The accumulation of phytoalexins in the interaction between rice and *M. oryzae* has been also described (Hasegawa et al. 2010).

3.2 Defense priming

The defense priming phenomenon is defined as a induced physiological state in which plants are conditioned for the activation of defense in a more strong, quick and long lasting period of time to a stress (Conrath et al. 2006; Martinez-Medina et al. 2016). Priming, although being an integral part of the systemic acquired resistance and the induced systemic resistance, is an adaptive measure independent of the constitutive expression of defense mechanisms that cause no impact in the host plant energy status and development. Priming events can occur as a result of interindividual or interspecies communication, such as induced resistance mediated by rhizobacteria, mycorrhizal fungi, or virulent or avirulent pathogens or by natural or axenic compounds (Jung et al.

2009; Aranega-Bou et al. 2014; Gamir et al. 2014). Recent studies demonstrated that priming consists in an epigenetic compound that can be inherited transgenerationally (Pastor et al. 2013).

3.3 Methylation in plant immunity

Global gene expression changes induced by pathogen infection have been characterized in detail. More recently, studies have begun to analyze how chromatin structure affects the expression of defense genes (Ding and Wang 2015). The packing of DNA into fibers occludes the access of to components that regulate transcription, DNA repair, replication and recombination, therefore, chromatin relaxation is required for the proper gene access. Chromatin unfolding involves several processes, and among them are DNA methylation changes (Li and Reinberg 2011).

DNA methylation is a conserved form of epigenetic regulation involved in transcriptional gene silencing (TGS) and maintenance of genome integrity through transposon taming (Deleris et al. 2016). In eukaryotes, methylation of the fifth carbon of cytosine (5mC) modulates chromatin structure, hence, gene expression (Zilberman 2008). Plant DNA methyltransferases target cytosines located at both symmetric (CG or CHG; H corresponds to A, C or T) and asymmetric (CHH) sites and these methylation patterns can be propagated to progeny. Conversely, DNA methylation can be removed by the activity of 5mC glycosylases and demethylases. In general but not always, DNA methylation is associated with loss of gene expression.

Plant DNA methylation patterns become altered by pathogen infection, which, in turn, might fine tune the expression of defense genes. Also, DNA methylation can exert regulation of immune-responsive genes containing transposable elements or DNA repeats in their promoter regions, or can regulate immune-responsive genes containing intronic transposable elements (Deleris et al. 2016).

In addition to DNA, histones can also be methylated. Methylation of histone H3K4 at the nucleosomes of *WRKY70* stimulates SA-dependent defense responses, repressing JA-dependent responses. *Pseudomonas syringae* induces the expression of *WRKY70* as well as the reduction of H3K27me2 and the accumulation of H3K4m2 and H3K4me3 in *WRKY70* (Alvarez-Venegas et al. 2007). In other studies, hypomethylation was reported in *P.* syringae-infected Arabidopsis plants which was associated with decondensation of heterochromatin. At the structural level, hypomethylation may affect the stability of *NBS-LRR* genes, these genes being usually clustered in regions rich in transposable elements and repetitive sequences (Pavet et al. 2006). Methylation state is not related directly to plant resistance, but rather, the mechanisms are substantially more complexes. A recent study has demonstrated that the link between the methylation state and plant resistance is dependent of the pathosystem. For instance, the hyper-methylated *ros1* (*Repressor of Silencing 1*) mutant is more susceptible to the biotrophic oomycete *Hyaloperonospora arabidopsidis*, while this mutant shows resistance to the fungus *Plectosphaerella cucumerina* (Lopez-Sanchez et al. 2016).

3.4 RNA silencing and plant disease resistance

In plants, RNA silencing is an ancient mechanism that directly defends host cells against foreign nucleic acids, including transposons or nucleic acids from pathogens such as viruses. This defense is stimulated by double-strand RNA (dsRNA), resulted from the amplification of invasive RNA, which is processed by the host into the small RNA (sRNA) pathway. These siRNAs guide silencing at the transcriptional (mainly epigenetically repressing the transcription by DNA methylation) or post-transcriptional level (Brodersen and Voinnet 2006). Historically RNA silencing mechanisms have been considered not to be involved in PTI or ETI but evidences support that transcriptional and post-transcriptional changes mediated by endogenous RNAs are key regulators of PTI and ETI signalling.

4. Small RNAs in plant immunity

Small RNAs associated with post-transcriptional gene silencing were first discovered in plants in 1999 (Hamilton and Baulcombe 1999). Afterwards, genes encoding regulatory small RNAs have been identified in other kingdoms including fungi. Most small RNAs are 21-24 nt RNA molecules that are produced by a primary, long RNA transcript. DsRNA precursors can derive from overlapping sense, antisense transcripts, or from the synthesis of dsRNA from single-stranded RNA. Endogenous small RNAs in plants can be divided into two major classes: miRNA and small-interfering RNAs (siRNA).

Traditionally, studies about regulation of plant immunity focused at the transcriptional control of defense gene expression, however, nowadays several publications support that plants are able to use post-transcriptional regulation of gene expression as part of their immune responses through RNA-based mechanisms.

4.1 microRNAs (miRNAs) in plants

4.1.1 Biogenesis and mode of action

MicroRNAs (miRNAs) are small endogenous RNAs involved in post-transcriptional regulation of gene expression. Currently, there are 713 and 427 miRNAs annotated in rice and Arabidopsis, respectively (miRBase, release 21). MiRNA biogenesis starts with the transcription of *MIR* genes by RNA polymerase II into long primary RNA transcripts (pri-miRNA), which fold into hairpin structures (**Figure 9**). The pri-miRNAs are cleaved by RNAse III-like enzymes, typically DICER-like 1 (DCL1) in a two-step process to produce smaller stem-loop structures called precursor miRNAs (pre-miRNAs), which are subsequently processed to produce miRNA duplexes (miRNA/miRNA*, also named miRNA-5p/miRNA-3p)(**Figure 9**) (Rogers and Chen 2013a). Although DCL1 is the main DICER-like protein involved in miRNA biogenesis, DCL3 and DCL4 are also capable of processing miRNA precursor transcripts (Rajagopalan et al. 2006; Vazquez et al. 2008; Cuperus et al. 2011). The miRNA duplex is methylated at both strands by HUA ENHANCER 1 (HEN1) at its 3’ end to protect miRNAs from 3’-exonuclease degradation and 3’-uridylation (**Figure 9**). The duplex is then transferred to the cytoplasm by a nuclear membrane protein known as HASTY (HST), ortholog of mammalian Exportin 5, where a strand of mature miRNA binds AGO at the RNA-induced silencing complex (RISC). Then, miRNAs guide cleavage or translational repression of mRNA targets. AGO1 is the main argonaute involved in miRNA action, but in some cases it is possible to find the participation of AGO7 (Endo et al. 2013) and AGO2 (Zhang et al. 2011b). Other RNA-binding proteins necessary for plant small RNA biogenesis are the HYPONASTIC LEAVES 1 (HYL1), SERRATE (SE), NEGATIVE ON TATALESS 2 (NOT2) and DAWDLE (DDL) (Bartel et al. 2004; Kurihara and Watanabe 2004; Voinnet 2009; Arikit et al. 2013; Achkar et al. 2016) (**Figure 9**).

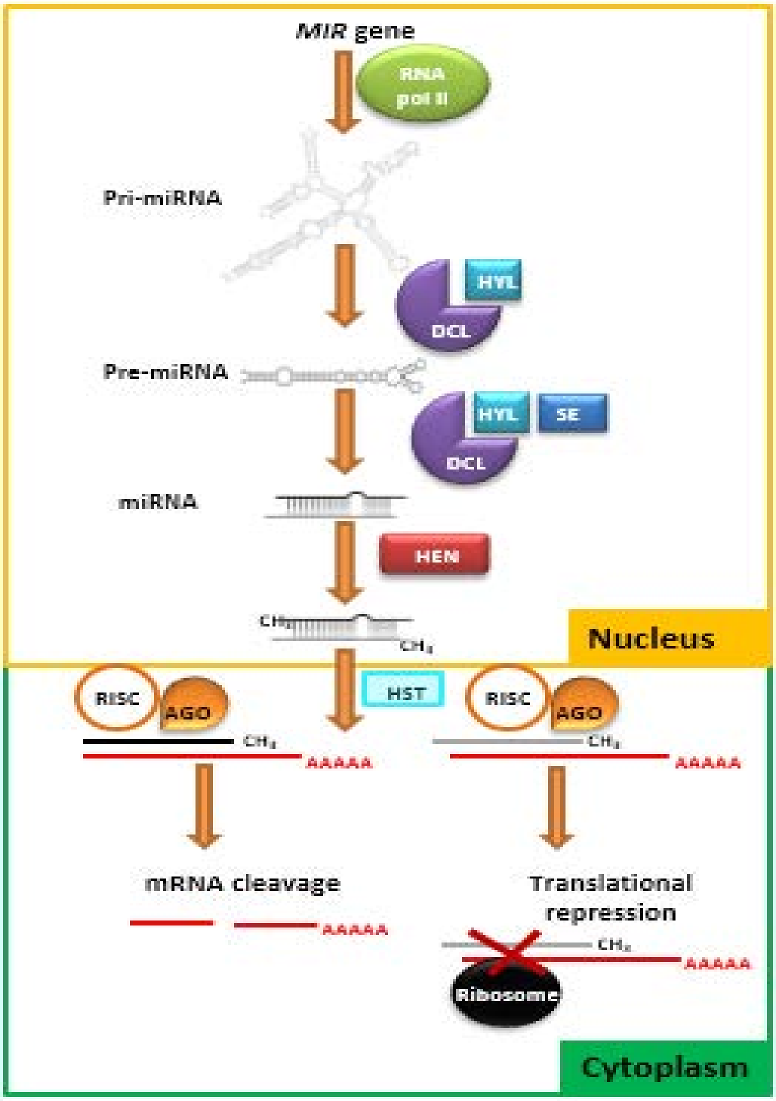

Figure 9. Biogenesis of miRNAs. *MIR* genes are transcribed into pri-miRNAs, which are processed by DCL, typically DCL1, in two steps generating pre-miRNAs and miRNA duplexes (miR/miR* or miRNA-5p/miRNA-3p). MiRNAs are methylated and transported to the cytoplasm where they are recognized by ARGONAUTE proteins, the core component of the RISC to execute messenger RNA degradation or translational repression. RNA pol II, RNA polymerase II; DCL, dicer-like; HYL, hyponastic leaves; SE, serrate; HEN, Hua enhancer 1; HST, Hasty; RISC, RNA-induced silencing complex; AGO, argonaute.

The vast majority of miRNA genes are species- or family-specific, suggesting rapid evolution and high turnover rate (Fahlgren et al. 2007). Based on their sequence, miRNAs are grouped in families consisting of different members designated by a letter after the miRNA number (e.g. miR398a, miR398b).

Plant miRNA precursors are more variable in length than their fungal or animal counterparts. In plants miRNA precursors can be divided into two main groups depending on their sequence and structure which determine their mode of biogenesis. One group harbors the precursors with 15-17 nt stem below the miRNA/miRNA*, which specifies the first cut of DCL1, and then a second cut occurs approximately 21 nt away from the first cleavage site realizing the mature miRNA (base to loop processing) (**Figure 10**). The second group contains precursors that are processed from loop to base, in some cases by several cuts instead of the usual two (Addo-Quaye et al. 2009; Bologna and Voinnet 2014) (**Figure 10**). It has been proposed that the evolutionary conservation of certain pattern sequences in miRNA precursor transcripts is what guides DCL1 to start de miRNA cleavage from base to loop or loop to base (Chorostecki et al. 2017) (**Figure 10**).

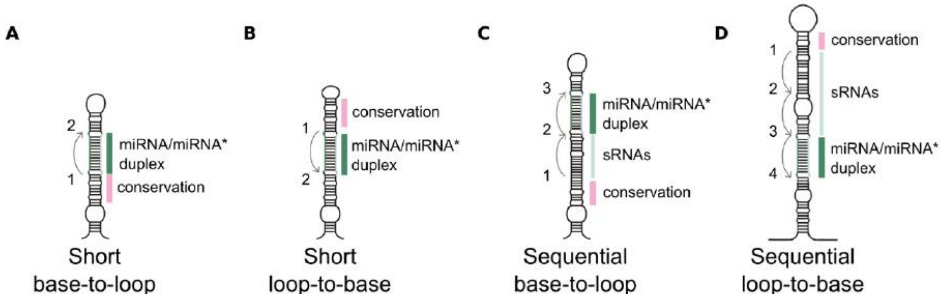

Figure 10. Orientation of miRNA precursor recognition and cleavage by DCL1. DCL1 can perform the typical two cleavages or act sequentially, producing miRNAs and other double stranded sRNAs. From Chorostecki et al 2017.

4.1.2 Polycistronic miRNAs

The evolution and diversification of miRNAs can be driven polyploidization (whole genome duplication), segmental duplications of subchromosomal regions, or tandem duplications (local duplications that involve only a few genes). Along with this, members of distinct miRNAs families have been found to locate in duplicated genomic regions in rice (Jiang et al. 2006). As result of these mechanisms miRNAs clusters have been identified in plants (e.g. miR166, miR169 and miR395) (Calviño and Messing 2013; Barik et al. 2014). Unlike animals, only 46 clustered miRNAs are registered in miRBase (release 21) in rice. Short distances (maximal distance of 3 Kb) between consecutive *MIRNA* loci in the same strand are a hallmark of clustered miRNAs in humans (Altuvia et al. 2005). Clustered miRNAs can be transcribed either independently or simultaneously into single polycistronic transcripts.

Polycistronic miRNAs fall into two categories: homologous polycistronic miRNAs if they contain copies of the same miRNA family member, and non-homologous polycistronic miRNAs if they contain unrelated miRNAs. Homologous and non-homologous polycistronic miRNAs have been recently identified in rice (Baldrich et al. 2016).

Besides their importance for miRNA evolution, duplications also contribute to the diversification of expression and functional properties of miRNAs. The simultaneous transcription of miRNAs in a same polycistronic precursor represents a new level of complexity in miRNA function controlling gene expression, as different mature miRNAs are controlled by an unique promoter and at the same time they can regulate from developmental patterns to stress responses.

4.1.3 Function of plant miRNAs

Many studies described the pivotal role of plant miRNA in the regulation of gene expression during many developmental processes including organ polarity, shoot and root development, morphogenesis, flowering and hormone signalling, among others (Palatnik et al. 2003; Chen 2004; Mallory et al. 2004; Rubio-Somoza and Weigel 2011). In rice, the activity of certain miRNAs has been shown to significantly control important agronomical traits, such as tiller growth, flowering and grain production (Miura et al. 2010; Wang et al. 2012; Zhang et al. 2013). Specific miRNAs have also been reported to play a role in adaptation to a variety of abiotic stresses, such as mineral nutrient deficiency, drought, cold and salinity (Chiou et al. 2006; Jagadeeswaran et al. 2009; Sunkar et al. 2012). The crucial role of miRNAs in disease resistance was first described in Arabidopsis (Navarro et al. 2006). Since then, many miRNAs have been shown to be regulated by pathogen infection (Shivaprasad et al. 2012; Campo et al. 2013; Li et al. 2014; Huang et al. 2016). MiRNAs can function as positive or negative regulators in PTI and ETI by targeting negative or positive defense regulators, respectively. Although a substantial fraction of the Arabidopsis and rice miRNA transcriptomes has been shown to be pathogen-responsive (Staiger et al. 2013), the exact role of most of these pathogen-regulated miRNAs in immunity remains elusive. Currently the functional role of miRNAs in plant disease resistance is, however, less studied than in plant development.

4.1.4 MiRNAs and bacterial infection

The involvement of miRNAs in PTI responses and pathogen resistance was first demonstrated in Arabidopsis where perception of flg22, a peptide derived from the general elicitor flagellin, causes an increase in miR393 accumulation which, in turn, negatively regulates auxin signalling by targeting auxin receptors (transport inhibitor response 1,TIR1; auxin signalling F-box protein 2, AFB2 and AFB3). MiR393-mediated repression of auxin signalling results in bacterial resistance (Navarro et al. 2006). This study clearly established a link between miRNAs, hormone signalling and immunity in plants. In addition miR393* (miR393-3p) is also involved in defense responses in Arabidopsis. MiR393* regulates MEM12, a Golgi-localized SNARE protein that modulates exocytosis of antimicrobial PR1 proteins (Zhang et al. 2011b). In rice, miR393 targets *OsTIR1* and *OsAFB2*, and overexpression of miR393 results in increased tillers, early flowering, reduced tolerance to salt and drought and hyposensitivity to auxin (Xia et al. 2012; Bian et al. 2012). Thus, miR393 appears to function in auxin signalling in both Arabidopsis and rice plants.

Besides miR393, miR160 and miR167 also target members of the auxin-responsive factor (ARF) family involved in auxin signalling (Rhoades et al. 2002). MiR393 and miR167 have also been described to be regulated during the infection of *Agrobacterium tumefaciens*, a bacterium of general interest because its widespread use for transferring genes into plant genomes. An oncogenic strain of *A. tumefaciens* induced miR393 and miR167 at the infiltration zones, whereas the strain lacking tumorigenic properties did not induce these miRNAs (Dunoyer et al. 2006).

Another miRNA whose accumulation is altered during bacterial infection in Arabidopsis is miR398 (Jagadeeswaran et al. 2009), which targets transcripts encoding Cu/Zn superoxide dismutases (CSD1 and CSD2). To investigate the effect of bacterial infection on miR398 accumulation, plants were infected with both virulent (*Pst* DC3000) and avirulent (*Pst* DC3000 carrying effector *avrRpt2* or *avrRpm1*). MiR398 was down-regulated by infection with *Pst* DC3000*(avrRpt2)* and *Pst* DC3000*(avrRpm1)*, thereby there were increasing levels of *CSD1* and *CSD2* to alleviate the oxidative stress promoted by the infection, reinforcing plant defense.

MiR390 is also misregulated during *P. syringae* infection in Arabidopsis. The expression of miR390 is down-regulated during the bacterial infection and consequently, the accumulation of miR390 target transcripts (*TAS3*) increases. This triggers the production of ta-siRNAs (trans-acting siRNAs) that regulate the expression of *ARF3* and *ARF4*, genes involved in auxin signalling (Zhang et al. 2011a). Not only auxin signalling, but other hormone signalling pathways can be under miRNA regulation. For instance, the jasmonic acid biosynthesis pathway is regulated by miR319 and miR159 whereas the ABA signalling pathway is regulated by miR159 (Curaba et al. 2014). In other studies, Arabidopsis plants overexpressing either miR400 or miR844 showed much severe disease symptoms compared to the wild-type during *P. syringae* infection. MiR400 triggers the cleavage of transcripts encoding pentatricopeptide repeat (PPR) proteins, whereas miR844 targets cytidinephosphate diacylglycerol synthase 3 (CDS3) (Park et al. 2014; Lee et al. 2015). Other miRNA participating in Arabidopsis immunity are miR160 and miR773. MiR160 positive regulated the accumulation of callose, conversely miR773 negatively regulates the PAMP-induced callose deposition and disease resistance to *P. syringae* (Li et al. 2010b).

MiRNAs are important also for ETI responses. A miRNA superfamily comprising miR482 and miR2118 targets *NBS-LRR* genes in tomato. In this way, their expression allows the simultaneous silencing of multiple *R* gene family members through the generation of siRNAs (Shivaprasad et al. 2012b). Other miRNAs targeting *NBS-LRR* genes are miR2109, miR472, miR1510 in *Medicago truncatula* (Zhai et al. 2011) and miR472 in Arabidopsis (Boccara et al. 2014).

4.1.5 MiRNAs and viral infection

Alterations in the expression of small RNAs have been reported during viral infection in many plants. In *Solanum lycopersicum*, for exemple, it has been demonstrated that miR482 is down-regulated during *Turnip Crinkle Virus* (TCV), *Cucumber Mosaic Virus* (CMV) and *Tobacco Rattle Virus* (TRV) infection, letting to an enhance of its target, a *NBS-LRR* gene (Shivaprasad et al. 2012b). Other miRNAs targeting *NBS-LRR* genes described during viral infections are miR158 and miR885 in the pathosystem *Brassica napus* / *Turnip Mosaic Virus* (TuMV), and miR6019 and miR6020 in the pathosystems *Nicothiana tabacum* / *Tobacco Mosaic Virus* (TMV) (Li et al. 2011; Zhai et al. 2011). Two common viral infections in rice are caused by *Rice Stripe Virus* (RSV) and *Rice Dwarf Virus* (RDV). Some miRNA found to be regulated by these viruses are miR160, miR166, miR167, miR168 miR171 and miR396, although some can respond to RSV but do not to RDV, and the other way around (Du et al. 2011; Wu et al. 2015).

4.1.6 MiRNAs and fungal infection

Plants can defend themselves from pathogenic fungi using the RNA silencing strategy. Some of the miRNAs previously mentioned functioning in the response to bacterial pathogens also play a role in the plant response to fungal infection (e.g miR160, miR393 and miR396). MiR160 and miR393 (targeting *ARF10* and *TIR1* respectively) are up-regulated in *Manihot esculenta* during *Colletotrichum gloeosporioides* infection for the modulation of auxin signalling, and subsequent activation of plant defense responses (Pinweha et al. 2015). Conversely, miR396a appears to function as a negative regulator of plant disease resistance during the infection of *Solanaceae* species (e.g. tomato) with the oomycete *Phytophthora infestans*. Here miR396 overexpression causes a reduction in *growth regulating factor* (*GRF*) transcripts, and transgenic plants are more susceptible to the pathogen (Chen et al. 2015). In Arabidopsis, miR396 negatively regulates PTI responses to fungal pathogens as *Plectosphaerella cucumerina¸ Fusarium oxysporum* and *Colletotrichum higginsianum* (Soto-Suárez et al. 2017). In other studies in cotton, it was reported that *Verticillium dahliae* promotes the down-regulation of miR482, which in turn induces the expression of R genes (Zhu et al. 2013).

4.2 MiRNAs controlling immunity to the rice blast fungus

Despite the great efforts for the characterization of miRNA populations in different rice tissues, and in response to biotic stress, the exact role of most of these pathogen-regulated miRNAs in rice immunity remains elusive. Sequencing of small RNAs populations revealed dynamic alterations in the expression of a large number of miRNAs in response to blast fungus or treatment with elicitors prepared from this fungus (Campo et al. 2013; Li et al. 2014; Baldrich et al. 2015). An important number of *M. oryzae* regulated miRNAs are known to be involved in small RNA pathways. Among them are miR162 and miR168, targeting *DCL1* and *AGO1* respectively. This observation suggests a pathogen-regulation of the small RNA machinery itself. However, only a few miRNAs have been functionally characterized in the interaction of rice with the fungal pathogen *M. oryzae*.

Distinct miRNAs for which a function in blast disease resistance has been demonstrated are miR160a, miR169, miR398b and miR7695 (Campo et al. 2013; Li et al. 2014; Li et al. 2017c). Overexpression of miR160a or miR398b results in increased H_2O_2 accumulation at the infection site and up-regulation of defense related gene expression, promoting enhanced resistance to blast disease (Li et al. 2014). Transgenic rice lines overexpressing miR169a are hyper-susceptible to *M. oryzae* which is associated to reduced expression of defende genes (Li et al. 2017). MiR7695 is a recent evolved, rice specific miRNA that targets an alternative spliced transcript of the *Natural resistance-associated macrophage protein 6* (*OsNramp*) gene. Overexpression of miR7695 has been shown to confer resistance to infection by the rice blast fungus.

Because miRNAs provide a quantitative regulation of target gene expression, the observed dynamic regulation of the accumulation of rice miRNAs during pathogen infection would provide the fine-tuning of gene expression in physiological processes contributing to disease resistance. In order to understand the specific function of pathogen-regulated miRNAs and their contribution to disease resistance further investigation is needed.

4.3 Other small interfering RNAs (siRNAs) in plants

SiRNAs originate from different types of double-stranded RNAs (dsRNA), coming from sense and antisense complementary transcripts, inverted-repeat sequences of unrelated RNAs (for instance, viral RNAs), or by the activity of RNA-dependent RNA polimerases (RDRs).

There are different classes of siRNAs: heterochromatic siRNAs (hc-siRNA), phased secondary siRNAs (phasi-RNA) and natural antisense siRNAs (nat-siRNA). The 24 nt class of siRNAs is the most abundant one (Xie et al. 2004). Production of each class of small RNA has its own requirements. The biogenesis of a hc-siRNA requires transcription by RNA polymerase V, followed by dsRNA synthesis by RDR2 and processing by DCL3 (Ausin et al. 2012). By contrast, tasi-RNA and phasi-RNA biogenesis requires targeting by 21nt miRNAs of mRNA transcripts. Cleaved transcripts function as templates for dsRNA synthesis by RDR6 and for the production of 21 and 22nt siRNAs by DCL2 or DCL4. Secondary siRNAs are often phased, so their first nucleotide occurs every 21 or 22nt from the miRNA-cleavage site (Liu et al. 2007a; Wang et al. 2011b; Song et al. 2012; Ou-Yang et al. 2013; Fei et al. 2016; Komiya 2016). Finally nat-siRNA can be transcribed by RNA polymerases II or V, the action of RDR2 or RDR6 and the processing of DCL1, DCL2 or DCL3 to produce 21-24 nt RNAs (Axtell 2013).

SiRNAs are regulators of gene expression at a transcriptional and post-transcriptional level. Whereas, heterochromatic-siRNAs act at transcriptional level controlling the silencing of certain genes through DNA methylation (Volpe 2002). Phasi-RNA and nat-siRNA guide mRNA degradation, similar to miRNAs.

4.4 Contribution of small RNA pathway components in plant immunity

Different components of small RNA biogenesis (such as DCLs) and functioning (such as AGOs) have been reported to function in plant immunity. There are four DCLs encoded in the *Arabidopsis thaliana* genome (DCL1–4) and five (DCL1-DCL5) DCLs in rice (*Oryza sativa*) (Gasciolli et al. 2005; Kapoor et al. 2008; Liu et al. 2009). DCL1 typically generates miRNAs, but can also produce other endogenous siRNA. As for DCL2, DCL3 and DCL4 they are involved in the production of siRNA species.

In Arabidopsis *dcl1* mutants were found to be more susceptible to the bacterial pathogen *P. syringae* pv. *tomato* DC3000 supporting that DCL1 is a positive regulator of PTI (Navarro et al. 2008). Contrary to this, in rice silencing of *dcl1* by RNA interference has been shown to have a positive impact on rice resistance to *M. oryzae* infection (Zhang et al. 2015). Thus, differences on the behavior of DCL1 might be dependent on the host and pathogen. On the other hand, the *dcl4* Arabidopsis mutant displays enhanced susceptibility to the fungal pathogen *Verticillium dahliae,* the causal agent of the verticillium wilt disease, which suggest that DCL4 plays a positive role in the host defense response (Ellendorff et al. 2009). The expression of DCL proteins is also regulated by viral infections (Blevins et al. 2006).

Evidence show that HYL1, a protein associated to DCL1, is involved in antibacterial defense (Katiyar-Agarwal et al. 2007). The mutant *hyl1* is more susceptible to *P. syringae* and also deficient in the accumulation of two siRNAs (nat-siRNAATGB2 and AtlsiRNA-1) which appear to positively regulate bacterial resistance (Katiyar-Agarwal et al. 2006; Katiyar-Agarwal et al. 2007).

AGO proteins, components of the small RNA functioning pathway, also contributes to plant immunity which is supported by the observation that

Arabidopsis *ago1* mutant plants displayed attenuated PTI responses (Li et al. 2010b). Arabidopsis AGO2 also plays a role in antibacterial immune responses. Thus, AGO2 expression is highly induced by infection with *P. syringae,* and *ago2* mutants display enhanced susceptibility to this pathogen (Zhang et al. 2011b). In other studies, *ago4* mutant exhibited susceptibility to this bacterial pathogen in Arabidopsis (Agorio and Vera 2007) and a mutation in AGO7 weakly attenuates ETI (Katiyar-Agarwal et al. 2007; Li et al. 2010a; Zhang et al. 2011b).

This piece of evidence supports the idea that components of the small RNA biogenesis and functioning contribute to plant immunity.

5. Applications of miRNAs to plant disease resistance

Environmental stress is the most important force that adversely affects plant growth, development, productivity and quality of crops. To withstand this natural force, in addition to the need of food production due rapid population growth, crop production should be significantly improved and increased in a sustainable manner in the future. Modulation in the expression of certain miRNAs has been demonstrated to be useful in the control of agronomic traits in rice. For instance, miR397 overexpression improves rice yield by increasing grain size and promoting panicle branching (Zhang et al. 2013). As mentioned previously, disease resistant transgenic rice have been produced by overexpressing miR160, miR169, miR398 and miR7695 (Campo et al. 2013b; Li et al. 2014; Li et al. 2017) supporting the potential of miRNA sequences to prevent disease resistance. Furthermore, miRNAs can be also involved in processes associated to plant growth and development. For this reason, it is important to elucidate the mechanisms that are regulated by miRNAs potentially contributing to plant resistance, and links with regulatory networks controlled by miRNAs.

Both, artificial miRNAs (amiRNAs) and the miRNA target mimicry technologies have proven to be useful tools to decipher the function of genes of interest and should have applicability for improvement of plant disease resistance. Designed specifically artificial miRNAs (amiRNA) can be created by exchanging the miRNA duplex sequence within a miRNA precursor with a sequence designed to match the target gene. The artificial target mimics technology is based on a mechanism that plants use naturally to negatively regulate the function of specific miRNAs (Franco-Zorrilla et al. 2007). Despite all these techniques have been successfully used in different plant species, there are still limitations in utilizing these strategies for crop protection due to social concerns that transgene methodologies trigger. Scientific efforts are still necessary for the safety assessment of transgenic plants. In this respect, genome editing might be of special interest, and TALEN and CRISPR/Cas9 technologies have emerged as an alternative to classical transgenic methods (Miao et al. 2013; Xing et al. 2014; Endo et al. 2015). These technologies are able to cause targeted changes in the plant genome and allow generating mutant plants free of exogenous DNA.

Although miRNA-based techniques represent important tools for the study of gene function in plant immunity, their efficacy may vary, and case-by-case studies are required. Finally, field trials should be carried out in order to ensure that results obtained on miRNA regulation in greenhouse conditions are conserved when plants are exposed to natural conditions.

Objectives

Objectives

The main objective of this PhD thesis was to generate new knowledge about miRNAs contributing to disease resistance in plants, as well as on the contribution of DCL1 (DICER-LIKE1), a key component in miRNA biogenesis, to plant immunity. While the function of miRNAs in controlling developmental processes is well documented, there is a lack of information on the role of miRNAs in plant immunity.

The specific objectives were the following:

1. To determine the function of polycistronic miRNAs in rice immunity. This study focused on three polycistronic miRNAs previously identified in our group: miR166k-166h, miR1861b-1861c and miR5534a-5534b. Of them, miR166k-166h has been studied in more detail. Results obtained in this study are presented in Chapter I.

2. To investigate the contribution of DCL1a in rice immunity. The RNase III enzyme DCL1 is involved in the processing of miRNA precursors to generate mature miRNAs. Results are presented in Chapter II.

3. To elucidate the function of miR773 in the immune response of *Arabidopsis thaliana* to fungal pathogens.

To accomplish these objectives, the following pathosystems have been used:

Objectives 1 and 2. Interaction of *O. sativa* (*japonica* cultivar Tainung 67) with the rice blast fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*), a hemibiotrophic fungus. The fungus *Fusarium fujikuroi* (*anamorph* stage of *Gibberella fujikuroi)* was also used in studies to investigate the function of DCL1 in rice immunity (Objective 2).

Objective 3. Interaction of *Arabidopsis thaliana* (Col-0) with the fungal pathogens:
i) *Plectosphaerella cucumerina* (previously known as *Fusarium tabacinum*; anamorph *Plectosporium tabacinum*), necrotrophic fungus.
ii) *Fusarium oxysporum* f. sp. *conglutinans*, hemibiotrophic fungus.
iii) *Colletotrichum higginsianum*, hemibiotrophic fungus.

Chapter I

Rice polycistronic miR166k-166h triggers *Magnaporthe oryzae* resistance through ethylene signalling

Abstract

MicroRNAs (miRNAs) are small RNAs acting as regulators of gene expression at the post-transcriptional level. In plants, most miRNAs are generated from independent transcriptional units, and only a few polycistronic miRNAs have been described. miR166 is a conserved miRNA in plants targeting the *HD-ZIP III* transcription factor genes. We show that a polycistronic miRNA comprising two miR166 family members, miR166k and miR166h, functions as a positive regulator in rice immunity against the blast fungus *Magnaporthe oryzae*. Rice plants in which *MIR166k-166h* expression is activated show enhanced resistance to *M. oryzae* infection which is associated to a superactivation of defence responses. Stronger induction of *MIR166k-166h* also occurs in resistant rice varieties compared to susceptible varieties. Notably, two novel target genes were identified for miR166, the *Ethylene Insensitive 2* (*EIN2*) and *ferredoxin-nitrite reductase* genes. The regulatory role of miRNA species encoded by the miR166h-166k polycistron results from the activity of the miR166* species generated from the miR166k-166h precursor, miR166k* and miR166h* (targeting *EIN2* and *ferredoxin nitrite reductase*, respectively). Our findings support a role for miR166 in regulating rice immunity through the control of *EIN2* expression and supports a versatile mechanism to simultaneously control the expression of different genes by polycistronic miRNAs.

Introduction

MicroRNAs are endogenous, small non-coding RNAs that mediate post-transcriptional gene silencing (Jones-Rhoades et al. 2006). Biogenesis of canonical miRNAs begins with the transcription of *MIR* genes by RNA polymerase II into long stem-loop miRNA precursors, or pri-miRNAs, that are sequentially processed by DICER-like to give rise to a pre-miRNA, and finally to double stranded miRNA duplexes, the miRNA-5p/miRNA-3p (also named miRNA/miRNA* duplexes) (Kurihara and Watanabe 2004), that will be transported to the cytoplasm, where the functional miRNA strand (either miRNA-5p or miRNA-3p) is incorporated into an ARGONAUTE1 (AGO1)-containing RNA-induced silencing complex (RISC) (Baumberger and Baulcombe 2005; Jones-Rhoades et al. 2006; Voinnet 2009; Rogers and Chen 2013a). MiRNAs guide post-transcriptional gene silencing through sequence-specific cleavage or translational repression of target transcripts (Llave et al. 2002a; Brodersen et al. 2008).

The crucial role of miRNAs in controlling a wide range of plant developmental processes and hormone signalling is well documented (Palatnik *et al.*, 2003; Chuck *et al.*, 2007; Chen, 2009; Rubio-Somoza and Weigel, 2011; Schommer *et al.*, 2012). Plant responses to abiotic stresses and nutrient deficiency are also regulated by miRNAs (Chiou et al. 2006; Sunkar et al. 2007; Ding et al. 2016; Gao et al. 2016). Although the accumulation of an important number of plant miRNAs has been shown to be altered during pathogen infection, our current knowledge of the biological roles of pathogen-regulated miRNAs in plant immunity is still limited and most of it based on the model plant Arabidopsis during interaction with *Pseudomonas syringae* (Staiger et al. 2013; Weiberg et al. 2014a; Fei et al. 2016b; Huang et al. 2016b; Kuan et al. 2016).

It is generally assumed that miRNAs have originated by duplication of preexisting protein-coding genes with subsequent mutations (Allen *et al.*, 2004; Rajagopalan *et al.*, 2006; Fahlgren *et al.*, 2007). The spontaneous evolution from hairpin structures in the genome, or derivation from transposable elements, has been also proposed to explain the origin of plant miRNAs (Felippes et al. 2008; Nozawa et al. 2012). It is generally assumed that whole genome duplication events and tandem or segmental duplications of *MIR* genes are responsible for the expansion and diversification of miRNA gene families in plants (Thiel *et al.,* 2009; Baldrich *et al.,* 2016). Unlike animals, where the occurrence of miRNA clusters is common, a few miRNA clusters have been described in plants (Boualem et al. 2008; Merchan et al. 2009; Calviño and Messing 2013; Barik et al. 2014; Baldrich et al. 2016). Clustered miRNAs can be independently or simultaneously transcribed as polycistronic transcripts. The precursor structures of the polycistronic miRNAs might contain copies of members belonging to the same miRNA family (homologous polycistron), or unrelated miRNAs (non-homologous polycistron). Most of our understanding on polycistronic miRNAs in plants comes from studies in Arabidopsis (Merchan et al. 2009).

MIR166 is a large gene family and miR166 clusters have been identified in various plant species, such as *Medicago truncatula, Oryza sativa*, among others (Boualem *et al.*, 2008; Zhang *et al.*, 2009; Barik *et al.*, 2014; Li *et al.*, 2017). Based on alterations in the accumulation of miR166 during abiotic stress, it has been proposed a function as modulators of the plant response to diverse abiotic stresses (Kitazumi et al. 2015; Akdogan et al. 2016; Hamza et al. 2016; Xu et al. 2016; Li et al. 2017b). Functional evidence for miR166 in adaptation to pathogen infection has not been, however, reported.

We recently reported the occurrence of various homologous and non-homologous polycistronic miRNAs in rice. Among them, a polycistronic miRNA comprising two miR166 family members, the miR166k-166h, was described. Expression profiling revealed that mature miRNAs generated from the miR166k-166h precursor are co-expressed in rice leaves (Baldrich *et al.,* 2016). Furthermore, the use of deep sequencing technologies revealed that different miR166 isoforms differentially respond to pathogen infection or elicitor treatment in rice, and also differentially accumulate in resistant and susceptible varieties upon *Magnaporthe oryzae* infection (Li *et al.* 2014, 2016). In this work, we present evidence supporting that activation of *MIR166k-166h* expression confers resistance to infection by the rice blast fungus *M. oryzae*. Disease resistance in rice mutant plants in which *MIR166k-166h* expression is activated is associated to a stronger induction of defence responses during pathogen infection. Moreover, we identified *EIN2* and *ferredoxin-nitrite reductase* as novel target genes for miR166k-5p and miR166h-5p, respectively. Overall, results here presented support that the miR166k-166h polycistronic miRNA plays a role in rice immunity.

Material and Methods

Biological material

Rice (*Oryza sativa*) plants were grown at 28ºC/22ºC under 16h light/8h dark cycle. The T-DNA insertion line for *MIR166k-166h* (M0110144) and wild-type genotype (*O. sativa japonica* cv Taining 67) was obtained from the Taiwan Rice Insertional Mutant collection (TRIM; http://www.trim.sinica.edu.tw). Genotyping of the TRIM mutant was carried out by PCR on genomic DNA using a T-DNA-specific primer located at the left border of the T-DNA and a primer located in the vicinity of the insertion site. PCR products were confirmed by DNA sequencing. Determination of the T-DNA copy number in the rice mutant was carried out by quantitative PCR (qPCR) using the monocopy *sucrose phosphate synthase gene* as the endogenous reference gene (primers are listed in **Supplemental Table 1**). The fungus *M. oryzae* (strain *Guy-11*) was grown on complete medium as previously described (Campos-Soriano *et al.,* 2012). Saber, TeQuin, Kanto 51, Maratelli and Vialone Nano were obtained from the germplasm seed bank of the Consiglio per la Ricerca e la Sperimentazione in Agricoltura (CRA-Rice Research Unit, Vercelli, Italy). The resistant genotypes are characterized by the presence of the resistance (R) genes *Pik* in Kanto51, and *Pib* in Saber and TeQing (Tacconi et al. 2010).

Infection assays and elicitor treatment

For infection assays, three weeks-old plants were spray-inoculated with a spore suspension of *M. oryzae* (5×10^5 spores/mL), or mock-inoculated. Development of disease symptoms was followed with time. Lesion area was determined using digital imaging software (Assess 2.0, American Phytopathological Society). Three independent infection experiments were performed (at least 24 plants per genotype in each experiment) and statistically significant differences among genotypes were determined by one-way ANOVA test. Quantification of fungal DNA in infected leaves was performed by qPCR using specific primers for the 28S DNA gene (Qi and Yang 2002). A standard curve was prepared using *M. oryzae* DNA.

For elicitor treatment, three week-old plants were sprayed with an elicitor suspension of *M. oryzae* (300 µg/mL) (Casacuberta et al. 1992), or mock solution.

ACC treatment

Three-week old rice plants were treated with ACC at a concentration of 50µM for 15min, 1h, 4h and 24h. Control plants were mock-inoculated.

RT-qPCR, stem-loop RT-PCR and 5'RACE PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from DNAse-treated total RNA (1 µg) with SuperScript III reverse transcriptase (Invitrogen GmbH) and oligo-dT. RT-qPCRs were performed using SYBR Green in Light Cycler 480 (Roche). The *cyclophilin 2* gene (Os02g02890) was used to normalize transcript levels in each sample. Stem-loop RT-qPCR was performed as described in Varkonyi-Gasic *et al.,* (2007).

The modified 5'-RNA ligase-mediated RACE was performed (Llave *et al.,* 2011). PCR amplified products were cloned and sequenced to check the cleavage in target genes.

Agroinfiltation in *Nicotiana benthamiana* leaves

For transient expression of *MIR166k-166h*, the genomic DNA fragment encompassing the entire miR166k-166h precursor was obtained by PCR from genomic DNA and cloned into the pC5300 vector under the control of the maize ubiquitin promoter. The *OsEIN2a* cDNA sequence cloned into the pC2300 expression vector designed for the production of C-terminal GFP-tagged fusion proteins under the control of the 35S Cauliflower Mosaic Virus promoter was ceded by Dr. J.S Zhang (Ma et al. 2013). Plasmid constructs were introduced into the *Agrobacterium tumefaciens* EHA105 strain. As a negative control, the empty vector was used. The *N. benthamiana RDR6-IR* line deficient for expression of the RNA-dependent RNA polimerase 6 was used as host plant (kindly provided by Dr. D. Baulcombe) (Schwach et al. 2005). Constructs harboring the miR166k-166h precursor or the *EIN2-GFP*, either alone or in combination, were agroinfiltrated in *N.benthamiana* leaves, and their expression was monitored at 2 days after agroinfiltration.

Small RNA Northern blot of the agroinfiltrated leaves was carried out using the small RNA fraction obtained from total RNA (200 µg). The oligonucleotide complementary to the miR166 sequence (**Supplemental Table 1**) was labeled with digoxigenin using the DIG oligonucleotide 3'-End Labeling kit (Roche). For detection of the EIN2-GFP protein, total protein extracts (50 µg) were subjected to SDS-PAGE (12.5% gels) and probed with an anti-GFP antibody.

Results

MIR166k-166h activation results in enhanced resistance to infection by the rice blast fungus *M. oryzae*

In plants, the miR166 family comprises an important number of miR166 species transcribed as independent units. The rice genome contains 11 loci encoding monocistronic miR166s (miR166a, miR166b, miR166c, miR166d, miR166e, miR166f, miR166g, miR166i, miR166j, miR166l and miR166m; miRBase release 21) which are distributed on 7 chromosomes (**Supplemental Fig. 1**). The presence of a polycistronic miR166 precursor at chromosome 2 encoding two miR166 family members, namely miR166k and miR166h, has also been reported (Barik et al. 2014; Baldrich et al. 2016). Mature miR166k/miR166k* and miR166h/miR166h* species locate in the 5' and 3' hairpin, respectively, in the miR166k-166h precursor structure (**Fig. 1A**, left panel). Loci encoding monocistronic transcripts for miR166k or miR166h are not identified in the rice genome.

In this work, a T-DNA tagged line (M0110144) carrying the T-DNA insertion upstream of the *MIR166k-166h* locus was identified produced in the Tainung 67 (TN67) background (Hsing *et al.,* 2007). Knowing that the T-DNA contains 8 copies of the *CaMV35* enhancer near the left border and that genes within 15 kb of the T-DNA left border and 5 kb of the right border might be activated by these enhancers, we hypothesized that this mutant might be an activation mutant for *MIR166k-166h*. Here, it is worth mentioning that mutant alleles for miRNAs are not easily found in insertional mutant collections due to the small size of *MIR* genes. The T-DNA insertion site was confirmed by PCR using combinations of gene- and T-DNA-specific primers followed by DNA sequencing of the PCR products (**Fig. 1A**, right panel). No homozygous *MIR166k-166h* plants were identified in PCR genotype screens. Most importantly, heterozygous mutant plants accumulated higher levels of miR166k-166h precursor transcripts, which correlated well with an increase in the accumulation of mature miR166k and miR166h sequences (**Fig. 1B**). These observations, confirmed that the TRIM mutant is an activation mutant for *MIR166k-166h* (hereinafter referred to as *miR166k-166h-Ac*). This mutant harbors a single copy of the T-DNA inserted in its genome (**Supplemental Table 2**).

The miR166 family is a highly conserved family of miRNAs in plants with conserved targets. The various miR166 family members target genes encoding

Class III homeodomain-leucine zipper (*HD-ZIP III*) transcription factor involved in many plant development processes (Emery et al. 2003; Itoh et al. 2008). In Arabidopsis, the *PHABULOSA* (*PHB*) and *PHABOLUTA* (*PHB*) genes are known to be targets of miR166 (Mallory et al. 2004b). Moreover, a miR166-mediated repression of the seed maturation program has been also described (Tang et al. 2012). Regarding monocistronic miR166s, it is well known that mature miR166 species directing cleavage of *HD ZIP III* transcripts originate from the 3’ arm of the stem-loop precursor structure (miR166h and miR166k). Accordingly, in this work we will refer to miR166k and miR166h as the miRNA strand targeting *HD-ZIP III* transcripts, whereas the complementary strand in the hairpin of the miR166k-166h precursor will be named as miR166k* or miR166h*. As it is shown in **Fig. 1B**, differences in the accumulation of miR166-related small RNAs between mutant and wild-type plants were more pronounced in the case of miR166k* species.

We considered the possibility that the expression of genes other than *MIR166k-166h* might be activated in the *miR166k-166h-Ac* mutant. An examination of the T-DNA insertional site revealed the presence of two genes, *OsSAUR12* (Os02g52990) and *Erwinia-induced protein* (Os02g53000) which were located upstream and downstream, respectively, of the T-DNA insertion site (**Supplemental Fig. 2A**). However, no alterations in the accumulation of either *OsSAUR12* or *Erwinia-induced protein* transcripts were observed in the *miR166k-166h-Ac* mutant (**Supplemental Fig. 2B**).

To investigate whether the miR166k-166h polycistronic miRNA plays a role in rice immunity, we performed disease assays. Wild-type and *miR166k-166h-Ac* plants were spray-inoculated with spores of the fungus *M. oryzae* ($5x10^5$ spores/mL), and disease symptoms were followed with time. Of interest, the *miR166k-166h-Ac* plants consistently showed reduced disease symptoms compared with wilt-type plants (**Fig. 1C**). Blast resistance was confirmed by quantification of fungal biomass and determination of lesion area in the infected leaves (**Fig. 1C**, right panels).

To get further insights into the mechanisms underlying disease resistance in the polycistronic miRNA activation lines, we evaluated the expression pattern of the defence-related genes *PBZ1* (*Probenazole-inducible 1*) and *PR1a* (*Pathogenesis-Related 1a*) in *miR166k-166h-Ac* plants at 24, 48 and 72 hours post-inoculation with *M. oryzae* spores. The *PBZ1* (a member of the *PR10* family of *PR* genes) and *PR1a* genes serve as markers for the activation of the defence response to pathogen infection, including *M. oryzae* infection (Midoh and Iwata 1996; Agrawal et al. 2001). As expected, fungal infection induced *PR1a* and *PBZ1* expression in wild-type plants. Importantly, transcripts of these defence genes accumulated to higher levels in *M. oryzae*-inoculated *miR166k-166h-Ac* than in wild-type plants at all time points of infection (**Fig. 1D**). These findings support that the miR166k-166h mutant respond more strongly to pathogen challenge. The observed superinduction of defence genes during fungal infection is consistent with the phenotype of disease resistance in *miR166k-166h-Ac* plants.

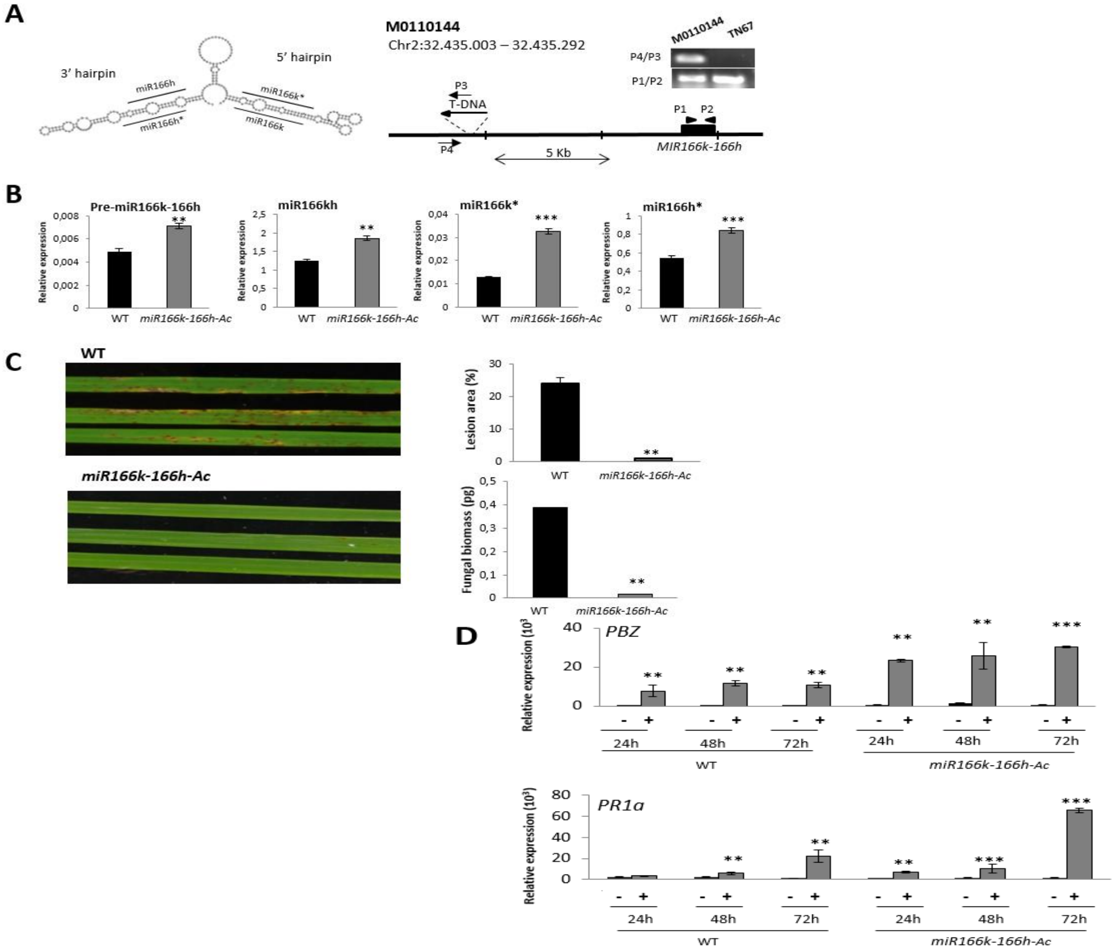

Figure 1. Characterization of polycistronic miR166k-166h activation mutant. A. Structure of the miR166k-166h precursor and location of mature miR166 sequences (left panel). Genotyping of the *miR166k-166h-Ac* (M0110144) by PCR (right panel). Arrows indicate the position of primers used for genotyping. **B.** Accumulation of miR166k-166h precursor transcripts and mature miR166 sequences in wild-type (TN67) and *miR166k-166h-Ac* mutant plants determined by RT-qPCR and stem-loop RT-qPCR, respectively. Note that the stem-loop RT-qPCR does not discriminate among miR166k and miR166h sequences. **C.** Phenotype of wild-type and *miR166k-166-Ac* mutant plants at 7 days post-inoculation with *M. oryzae* spores (5 x 10^5 spores/mL). Percentage of leaf area affected by blast lesions was determined by image analysis (APS Assess 2.0) (right upper panel). Quantification of *M. oryzae* DNA was determined by qPCR using specific primers of the *M. oryzae* 28S gene (right lower panel). **D.** Expression of the defence marker genes *OsPBZ* and *OsPR1a* in wild-type and *miR166k-166h-Ac* mutant plants that have been mock-inoculated or inoculated with *M. oryzae* spores. Histograms in B, C and D

show the mean ± SD (***, $P \leq 0.001$; **, $P \leq 0.01$, ANOVA test; *M. oryzae*-inoculated *vs* mock-inoculated).

*MIR166k-166*h expression during fungal infection and treatment with elicitors

Given that activation of *MIR166k-166h* has an effect on disease resistance, we sought to investigate whether *MIR166k-166h* expression is itself regulated during the normal host response to infection. Since the rice genome does not contain monocistronic miR166k and miR166h loci, the miR166k and miR166h mature sequences accumulating in rice leaves are expected to be generated from the polycistronic miR166k-166h precursor. There was a general and clear increase in the accumulation of miR166k-166h precursor transcripts at 24, 48 and 72 hpi, consistent with increased accumulation of mature miR166k and miR166h species (both miRNA and miRNA*) in *M.oryzae*-inoculated leaves of wild-type plants compared with non-inoculated leaves (**Fig. 2A**). Accumulation of precursor and mature miR166 sequences also increased in response to treatment with a crude preparation of elicitors (**Fig. 2B**). We also noticed that elicitor treatment results in faster and stronger induction in the accumulation of miR166k* and miR166h* species compared to miR166 species (miR166k and miR166h). Induction of marker genes of defence activation, confirmed that the host plant detects and responds to elicitor treatment (**Supplemental Fig. 3**).

MIR166k-166h expression was examined in disease resistant (Kanto 51, Saber and TeQing) and susceptible (Vialone Nano and Maratelli) rice varieties. As it is shown in **Fig. 2C**, *M. oryzae* infection induces *MIR166k-166h* expression in the resistant rice genotypes here assayed but its expression is barely affected by *M. oryzae* infection in the susceptible cultivars Maratelli and Vialone Nano.

From these results it is concluded that not only pathogen infection, but also treatment with fungal elicitors, results in up-regulation of *MIR166k-166h* expression, thus, suggesting a role of this polycistronic miRNA in PTI. Induction of *MIR166k-166h* expression appears to occur in resistant, but not in susceptible rice cultivars.

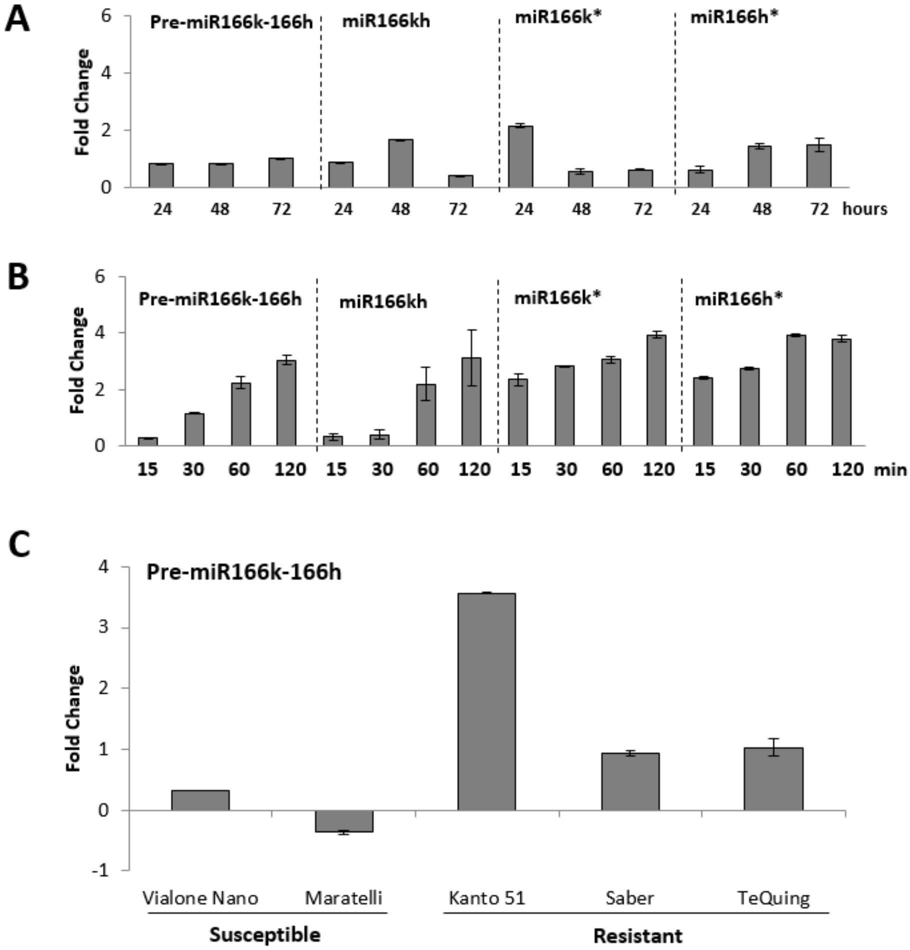

Figure 2. *MIR166k-166h* expression in response to *M. oryzae* infection. Accumulation of miR166k-166h precursor and mature miR166 sequences was performed by RT-qPCR analysis in wild-type plants. Fold-induction of gene expression (*M. oryzae* or elicitor-treated *vs* mock-inoculated) is shown. Three independent experiments were carried out (P-value ≤ 0.05, ANOVA test). A. Expression at different times after inoculation with *M. oryzae* spores ($5x10^5$ spores/mL). B. Expression in response to treatment with *M. oryzae* elicitors (300 µg/mL). C. Expression of miR166k-166h precursor in susceptible and resistant rice varieties infected with *M. oryzae.*

Analysis of cis-regulatory elements in the *MIR166k-166h* promoter

The promoter region of genes often includes *cis*-acting regulatory elements responsible for pathogen-inducibility. Knowing that fungal infection and elicitors treatment induced *MIR166k-166h* expression, we scanned the *MIR166k-166h* promoter region for the presence of *cis*-regulatory elements related to biotic stress. The sequence upstream of the precursor structure for the miR166k-166h precursor was extracted from the NCBI database and the transcription start site (TSS) was identified by using the TSSP Softberry program for the identification of TSS in plants (http://linux1.softberry.com). *Cis*-acting elements present in the 1.6 Kb DNA region upstream of the TSS were searched using the PLACE database (http://www.dna.affrc.go.jp/PLACE). Interestingly, the *MIR166k-166h* promoter was found to contain an important number of *cis*-elements required for responses to pathogen infection or elicitor treatment (**Fig. 3**; **Supplemental Table 3**). Among them, we identified several W-boxes (TGAC core sequences), such as WBOXATNPR1 (TTGAC), ERE (Elicitor Responsive Element; TTCAGG), WRKY710S (TGAC), and WBOXNTERF3 (TGACY) *cis*-elements (**Fig. 3**). These regulatory *cis*-elements are the binding sites for SA-induced WRKY transcription factors and are also found in many pathogen- and elicitor-responsive genes. The SEBF regulatory element (YTGTCWC), initially characterized in the promoter of the pathogen and elicitor inducible potato *PR-10a* gene, and later on in the promoter of several other *PR* genes, was also identified in the *MIR166k-166h* promoter. Other functional pathogen/elicitor-responsive elements identified in this analysis are the GT1-SCAM4 (GAAAAA) and PAL-responsive (CCGTCC) elements. Finally, regulatory elements associated with defence-related hormone signalling are also present in the *MIR166k-166h* promoter, such as the ethylene (ERELEE4, Ethylene-responsive element; AWTTCAAA) and the methyl jasmonic acid (T/G BOXPIN2, AACGTG) regulatory elements.

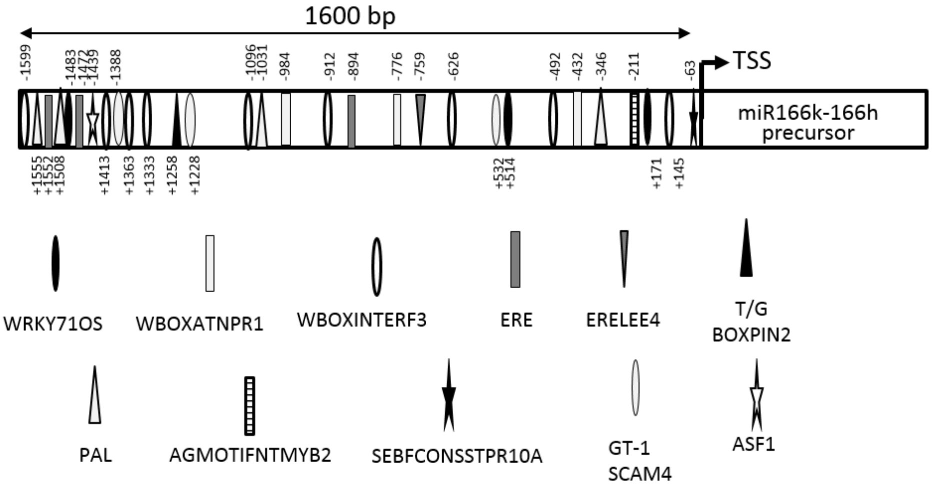

Figure 3. Structural features of the *MIR166k-166h* promoter. The location of known *cis*-acting elements related to biotic stress is shown (for details on *cis*-elements, see **Supplemental Table 2**). TSS, transcriptional start site.

Prediction and experimental validation of novel miR166 targets

As previously mentioned, t*argeting* of *HD-ZIP III* transcripts by *miR166* is highly *conserved* in plants (Jones-Rhoades and Bartel 2004; Nagasaki et al. 2007). In rice, five *HD-ZIP III* genes have been described, named as *Oshox9* (Os10g33960), *Oshox10* (Os03g01890), *Oshox29* (Os01g10320), *Oshox32* (Os03g43930) and *Oshox33* (Os12g41860) (Agalou et al. 2008). Of them, degradome analyses showed degradation tags indicative of miR166-mediated cleavage of *Oshox9*, *Oshox10*, *Oshox32* and *Oshox33*, supporting that these genes are real targets of miR166 in rice (Li et al. 2010a; Baldrich et al. 2015). As expected, RT-qPCR analysis revealed reduced levels of *Oshox9*, *Oshox10* and *Oshox32* in *miR166k-166h-Ac* mutant plants compared with wild-type plants (**Fig. 4A**) confirming functionality of mature miRNAs encoded by the polycistron. As for *Oshox29* and *Oshox33*, these genes were found to be expressed at very low level in wild-type plants, their expression not being significantly affected in the rice mutant compared to wild-type plants (**Fig. 4A**). On the other hand, genes other that *HD-ZIP III* have also been reported to be targeted by distinct miR166 family members. In particular, the rice *RDD1* (*rice Dof daily fluctuations 1*) transcription factor gene involved in nutrient uptake and accumulation was reported to be targeted by miR166b (Iwamoto and

Tagiri 2016). Very recently, a miR166-guided cleavage of *ATHB14-LIKE* transcripts encoding a homeobox-leucine zipper protein has been described in soybean (Li et al. 2017b).

Knowing that *MIR166k-166h* activation has an impact on blast resistance in rice, we considered the possibility that the phenotype of disease resistance observed in the *miR166k-166h-Ac* might be caused by the activity of miR166k* and/or miR166h* on novel, non-conserved target genes. Accordingly, a target prediction analysis was initially performed using the psRNATarget tool (http://plantgrn.noble.org/psRNATarget). Similar to other species, the target search predicted *HD-ZIP III* as target genes of miR166s encoded in the miR166k-166h polycistron. Interestingly, this computational prediction of target genes led to the identification of two putative target genes for the miR166k* or miR166h* sequences, *Ethylene-insensitive 2* (*EIN2*; containing the binding site for miR166k*) and *ferredoxin-nitrite reductase* (containing the binding site for miR166h*).

Contrary to Arabidopsis where EIN2 is encoded by a single gene, the rice genome possesses four *EIN2* genes, *OsEIN2.1* (also named as *MHZ7*; Os07g06130), *OsEIN2.2* (Os03g49400), *OsEIN2.3* (Os07g06300), and *OsEIN2.4* (Os07g06190) (Ma et al. 2013; Yang et al. 2015). Based on sequence homology, *OsEIN2* genes can be classified into two groups (*OsEIN2.1* and *OsEIN2.2*; *OsEIN2.3* and *OsEIN2.4*). The four *OsEIN2* genes have identical binding site for miR166k*. RT-qPCR analysis was carried out using gene-specific primers for *OsEIN2.1, OsEIN2.2* and *OsEIN2.3/4* (the high sequence homology between *OsEIN2.3* and *OsEIN2.4* did not allow the design of specific PCR primers for detection of *OsEIN2.3* and *OsEIN2.4* transcripts). This analysis, revealed down-regulation of *OsEIN2.1* and *OsEIN2.2* in *miR166k-166h-Ac* plants (**Fig. 4B**, left panel). Surprisingly, *OsEIN2.3* and *OsEIN2.4* transcripts accumulated at a higher level in the *miR166k-166h-Ac* mutant than in wild-type plants. The amount of uncleaved *OsEIN2* transcripts was diagnosed using PCR primers flanking the miR166k* cleavage site. Although the accumulation of uncleaved *EIN2* transcripts was notably reduced in the activation mutant, uncleaved transcripts still accumulated at an important level in these plants, likely due to the contribution of *EIN2.3/EIN2.4* transcripts (**Fig. 4B**, middle panel). These findings suggest the existence of complex regulatory mechanisms governing the expression of *OsEIN2* in which miR166k* directs cleavage of *OsEIN2.1* and *OsEIN2.2* transcripts while causing an increase in *EIN2.3/EIN2.4* transcripts.

As for *ferredoxin-nitrite reductase*, this is a single-copy gene in rice, its expression being down-regulated in *miR166k-166h-Ac* plants compared to wild-type plants (**Fig. 4B**, right panel). The observed inverse correlation between mature miR166k* levels and *EIN2.1* and *EIN2.2* transcripts in *miR166k-166h-Ac* plants was indicative of a miR166k*-mediated down-regulation of this particular *OsEIN2* family members. Equally, down-regulation of *ferredoxin-nitrite reductase* is consistent with a miR166h*-mediated regulation of this gene in the rice mutant.

These observations prompted us to investigate whether *EIN2* and *ferredoxin-nitrite reductase* genes are real target genes for miR166k-166h by performing RNA ligase-mediated 5’ RACE (5’-RACE). Sequencing of the 5’-RACE PCR clones revealed cleavage fragments of *EIN2* and *ferredoxin-nitrite reductase* transcripts. Transcripts for the two genes were found to be cleaved at the canonical position of miRNA/target mRNA pairing (between nucleotides 10 and 11 from the 5’ end of the miRNA) thus, supporting that *EIN2* and *ferredoxin-nitrite reductase* are indeed the target genes of miR166 in rice. We also noticed that miR166k*-guided cleavage products of *EIN2* and *ferredoxin-nitrite reductase* accumulated at a lower level in wild-type plants than in *miR166k-166h-Ac* plants (**Fig. 4C, D**, right panels). As expected, miR166-guided cleavage products of *hox32* were also identified by 5’-RACE (**Fig. 4E**).

Altogether, these results indicated that miR166 cleaves *EIN2* and *ferredoxin-nitrite reductase* transcripts. Furthermore, we show that mature miR166 sequences corresponding to the miRNA* strand of the miR166/miR166* duplex are functional.

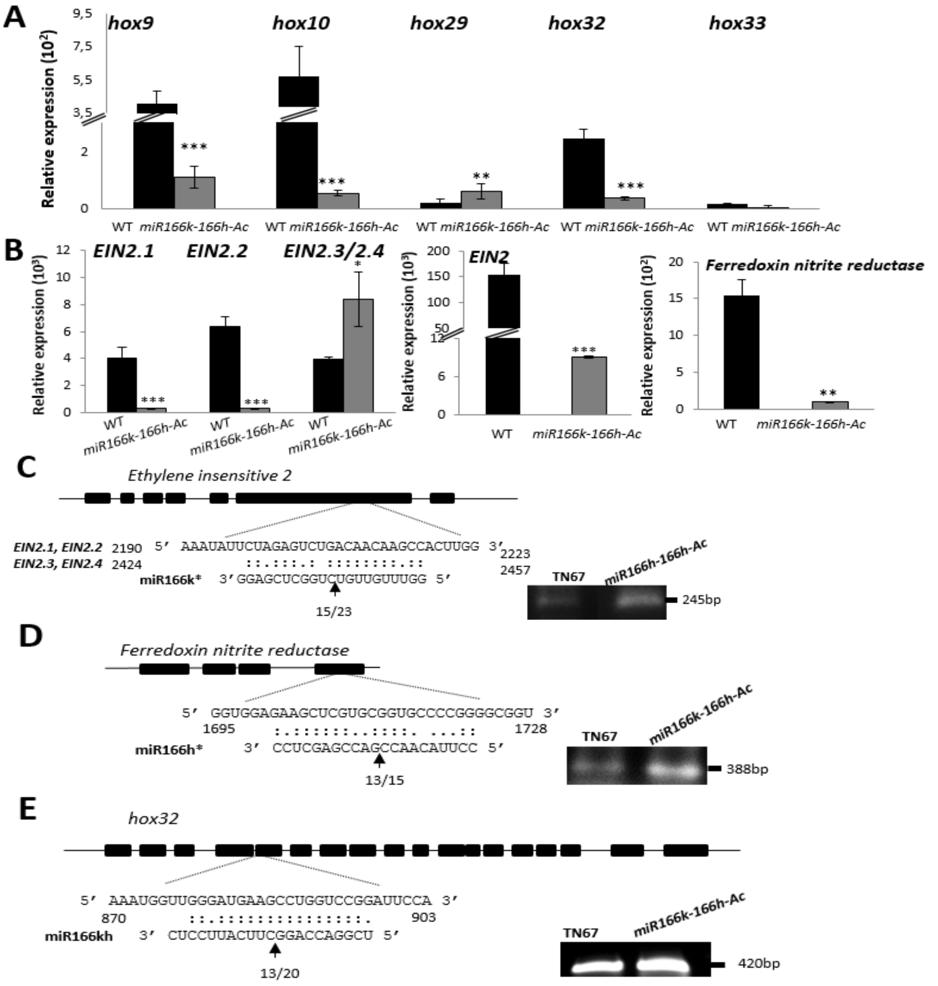

Figure 4. Expression of miR166 targets and identification of novel non-conserved target genes for miR166. A. Accumulation of *Oshox* transcripts encoding HD-ZIP III transcription factors in wild-type and miR166k-166h plants was determined by RT-qPCR analysis. **B.** Expression of *EIN2* and *ferredoxin-nitrite reductase* genes. Gene-specific PCR primers specific for *EIN2.1, EIN2.2* or *EIN2.3/2.4* (*both EIN2.3 and EIN2.4* sequences are closely related) were used (left panel). Primers spanning the miR166 target site in *EIN2* transcripts were also used (middle panel). Histograms (A, B) show the mean ± SD (***, P-value ≤ 0.001; **, P-value ≤ 0.01, ANOVA test). **C – E.** Experimental validation of miR166-mediated cleavage of *EIN2* (C), *ferredoxin-nitrite reductase* (B), and *Oshox32* (C) transcripts. Cleavage analysis was performed using 5' RACE using gene-specific primers and the resulting PCR products (right panels) were sequenced. The identified cleavage sites are indicated by an arrow, and the numbers below indicate the detected cleavage site of independent clones.

miR166k-166h-mediated cleavage of *EIN2* transcripts leads to reduced levels of EIN2 protein

Among the newly identified miR166 targets, *EIN2* is worth describing specifically. This gene is a central signal transducer in the ethylene signalling pathway in plants, and ethylene signalling is known to modulate plant immune responses in concerted action with other defence hormones, such as JA and SA (Solano and Ecker 1998; Jun et al. 2004; Glazebrook 2005; Bari and Jones 2009; Denancé et al. 2013; Ma et al. 2013).

We used agroinfiltration assays to further investigate the functional relationship between miR166h-166k activity and *EIN2* expression. The miR166k-166h precursor and a GFP-tagged *EIN2*-gene were co-expressed in *N. benthamiana* leaves. As controls, constructs bearing the empty vector, the miR166k-166h precursor alone, or the *EIN2-GFP* chimeric gene alone were also assayed in agroinfiltration experiments. Expression analysis revealed accumulation of precursor and mature miR166 sequences in leaves that have been agroinfiltrated with the miR166k-166h precursor (**Fig. 5A, EV and pre-miR166**; **Supplemental Fig. 4**), and confirmed proper processing of the polycistronic miR166k-166h precursor in *N. benthamiana*. Reduced levels of miR166k-166h transcripts were observed when the miR166k-166h precursor was co-expressed with *EIN2* compared with the miR166k-166h-only agroinfiltrated leaves (**Fig. 5A, left panel, *EIN2* and pre-miR166+*EIN2***). This observation could therefore suggest a possible *EIN2*-mediated negative feedback regulatory loop for *MIR166h-166k* expression in rice, an aspect that deserves further investigation. Northern blot analysis also showed accumulation of mature miR166k* in *N. benthamiana* leaves expressing *MIR166k-166h*, either alone or in combination with *EIN2* (**Fig. 5A**, right panel).

Co-expression of the miR166k-166h precursor with *EIN2-GFP* led to a clear reduction in *EIN2-GFP* transcripts compared with the *EIN2-GFP* gene alone (**Fig. 5B, left panel, *EIN2* and pre-miR166+*EIN2***). Moreover, reduction in *EIN2-GFP* transcripts was accompanied by a reduction in EIN2-GFP protein accumulation, as revealed by immunoblotting with an anti-GFP antibody (**Fig. 5B, right panel**). From these results, it is concluded that miR166k-166h cleaves *EIN2* in rice, and that results in a reduction in *EIN2* protein accumulation.

Finally, knowing *MIR166k-166h* expression is up-regulated by *M. oryzae* infection in wild-type plants (**Fig. 2**) and that *OsEIN2* is a target gene for miR166, we investigated *EIN2* expression during pathogen infection. Consistent with the up-regulation of *MIR166k-166h* during *M. oryzae* infection, *OsEIN2.1* and *OsEIN2.2* expression was down-regulated during the same period of infection, whereas by the same time, *OsEIN2.3/OsEIN2.4* expression was found to be up-regulated (**Fig. 5C**). Presumably, the increased levels of mature miR166k* and miR166h* species accumulating in *M. oryzae*-infected leaves would be responsible of down-regulation of *OsEIN2.1* and *OsEIN2.2* expression during pathogen infection. An inverse correlation was also found between *MIR166k-166h* and *ferredoxin-nitrite reductase* expression (**Fig. 5D**). Altogether, these results support that miR166k* produced from the miR166k-166h polycistronic precursor targets *OsEIN2* (e.g. *OsEIN2.1* and *OsEIN2.2*). A miR166k*-guided cleavage of *EIN2* transcripts causes a reduction in EIN2 protein accumulation.

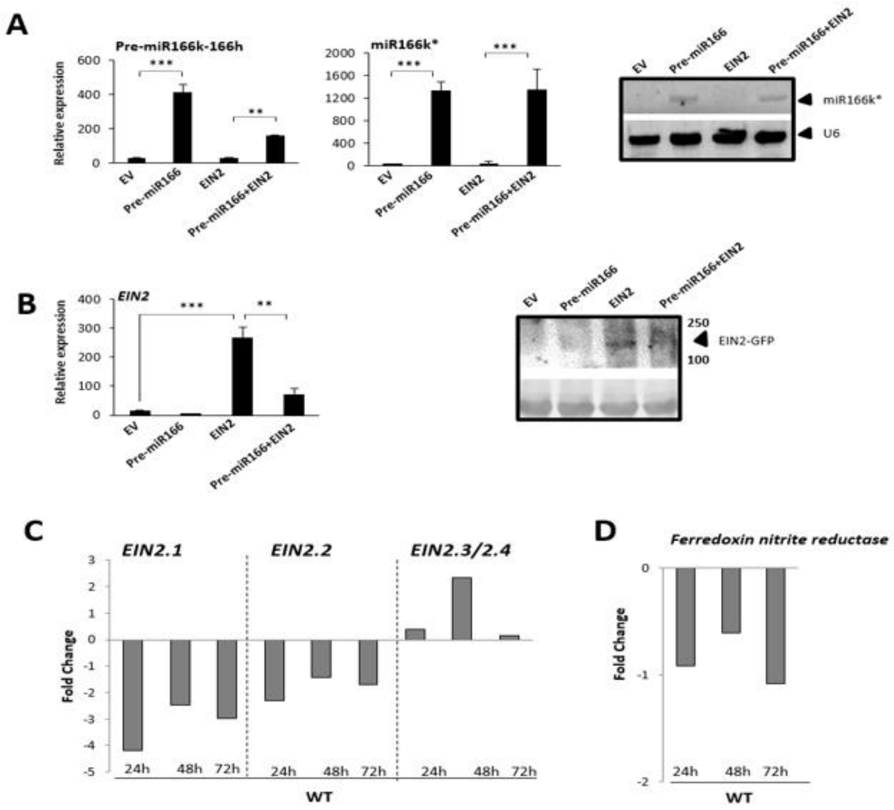

Figure 5. Validation of miR166k-166h-mediated down-regulation of *EIN2* by Agroinfiltration of *N. benthamiana*. Constructs harboring the miR166k-166h precursor only (pre-miR166), the *EIN2-GFP* only (EIN2), or the miR166k-166h precursor and *EIN2-GFP* were agroinfiltrated in *N. benthamina* leaves. The empty vector (EV) was used as control. Expression analyses were performed at 2 days after agroinfiltration. **A.** Accumulation of miR166k-166h precursor and miR166k* sequences was determined by RT-qPCR and ST-RT-qPCR, respectively (left and middle panels). Detection of mature miR166 sequences by small RNA Northern blot is shown on the right panel. For this, the small RNA fraction of agroinfiltrated leaf samples were hybridized with a 3’ end digoxigenin-labeled oligonucleotide sequence complementary to miR166k*. The same blot was stripped and rehybridized with the U6 probe. **B.** Accumulation of *EIN2a* transcripts and EIN2 protein in agroinfiltrated *N. benthamiana* leaves (left and right panels, respectively). Western blot analysis (right panel) was carried out using a polyclonal anti-GFP antibody. Ponceau staining of the protein blot is shown at the botton panel. **C**. *EIN2* expression in wild-type plants in response to *M. oryzae* infection. Data is presented as fold-change repression (*EIN2.1*, *EIN2.2*) or induction (*EIN2.3/2.4*) of gene expression (*M. oryzae*-inoculated *vs* mock-inoculated) at different times after inoculation of wild-type plants with *M. oryzae* spores. **D.** *Ferredoxine-nitrite reductase* expression in wild-type plants in response to *M. oryzae* infection.

Expression of ethylene signalling components in *miR166k-166h-Ac* plants

It is generally assumed that ethylene signalling plays an important role in basal defence response, and that *EIN2* is an essential regulator of ethylene signalling in plants. In rice, *OsEIN2* appears to function as a positive regulator of ethylene signalling as inferred from the observed repression of ethylene-inducible genes in *ein2* antisense rice plants (Jun et al. 2004). Here, it should be mentioned that the antisense construct used to silence *OsEIN2* expression in rice covered a 638 bp DNA fragment of the *EIN2.1* cDNA encompassing the C-terminal region of *EIN2*, a region with high sequence conservation among *OsEIN2* family members. Then, silencing of all four *OsEIN2* genes is expected in the *ein2* antisense plants previously described. On the other hand, accumulating evidence indicates that ethylene biosynthesis and signalling is required in rice for basal resistance against the blast fungus *M. oryzae* (Singh et al. 2004; Iwai et al. 2006; Helliwell et al. 2013, 2016a; Yang et al. 2017). Knowing this, a down-regulation of *OsEIN2* expression, a positive regulator of ethylene signalling, stands in apparent contradiction with ethylene signalling being necessary for resistance to the rice blast fungus.

Regarding ethylene signalling, it is generally assumed that phosphorylation of EIN2 prevents the signal from transduction in the absence of ethylene (Ju et al. 2012). Only in the presence of ethylene, EIN2 phosphorylation is reduced, and the C-terminal fragment of EIN2 is cleaved and translocated to the nucleus for activation of the downstream EIN3 and EIL1 transcriptional cascade. In addition, EIN2 and EIN3/EIL1 are regulated by proteasomal degradation through EBF1/2 (EIN3-binding F-box protein 1 and 2). EIN3 and EIL1 regulate the expression of ethylene-responsive proteins, including ERF1 (Ethylene Response Factor1) which, in turn, modulates the expression of various ethylene-responsive genes such as *PDF1.2* and *chitinase* genes (Kitajima and Sato 1999; Lorenzo et al. 2003; Bailey et al. 2009; Abiri et al. 2017; Solano and Ecker 1998).

To address the apparent contradiction of down-regulation of *OsEIN2* expression in *miR166h-166k-Ac* plants in the context of blast resistance*,* we investigated the expression of genes acting downstream of *EIN2* in the ethylene signalling pathway in these plants. Regarding *OsEIN3* and *OsEIL1*, we observed that both genes were up-regulated in *miR166k-166h-Ac* plants compared to wild-type plants. Similarly, *OsERF1* was found to be up-regulated in the *miR166k-166h-Ac* mutant, whereas *OsEBF2* was down-regulated in the rice mutant compared to wild-type plants (**Fig. 6A**). Consistent with up-regulation of *OsERF1*, the expression of ethylene-responsive defence genes as *PR3* and *PR4* (Rakwal et al. 2004; Wang et al. 2011b) was also up-regulated in *miR166k-166h-Ac* plants, for instance *PDF1.2* and *chitinase* genes, such as *CHIT8* and *CHIT14* (*PR3* family genes) and *WIP5* (*PR4* family member) (**Fig. 6B**). Thus, although miR166k-166h activation causes down-regulation of *OsEIN2.1* and *OsEIN2.2* expression, our data suggest that components in the pathway for ethylene signal transduction downstream of *OsEIN2* are induced in *miR166k-166h-Ac* plants, which would be in agreement with the resistance phenotype that is observed in these plants.

To provide clues for the function of the miR166k-166h polycistron in ethylene signalling we investigated whether miR166k-166h expression itself is regulated by ethylene in wild-type plants. For this, wild-type plants were treated with the ethylene precursor ACC and the accumulation of miR166k-166h precursor transcripts was determined after 15 min, 1, 4, and 24h of treatment. As it is shown in **Fig. 6C**, there was a clear increase in the accumulation of miR166k-166h precursor transcripts in ACC-treated wild-type plants.

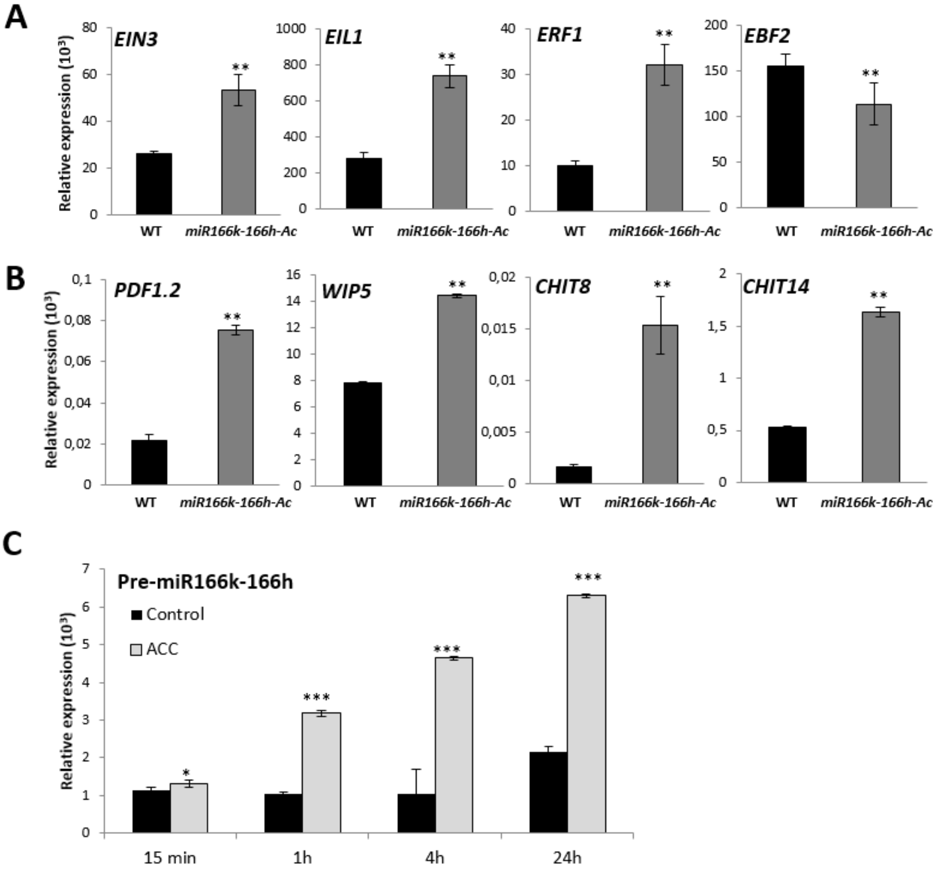

Figure 6. Expression of ethylene signalling components in the miR166k-166h activation mutant and *MIR166k-166h* expression in response to treatment with the ethylene precursor ACC. The accumulation of transcripts for genes acting downstream of *EIN2* was monitored by RT-qPCR using gene-specific PCR primers. A. Expression of *OsEIN3*, *OsEIL1*, *OsERF1* and *OsEBF2* in wild-type and *miR166k-166h-Ac* plants. B. Expression of *OsPDF1.2*, *OsWIP5* (*PR4* family), *OsCHIT8* and *OsCHIT14* (*PR3* family). C. Accumulation of miR166k-166h precursor transcripts in control and ACC-treated (50 µM) in wild-type plants. Histograms represent the mean ± SD (***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05; ANOVA test).

Discussion

Studies on plant immunity have traditionally focused on the transcriptional regulation of protein-coding genes. Because distinct miRNAs have been shown to function in PTI or ETI in different plant species, nowadays it is generally assumed that miRNAs are also important in plant immunity (Jagadeeswaran et al., 2009; Shivaprasad et al., 2012a; Campo et al., 2013; Boccara et al., 2014; Li et al., 2014; Baldrich and San Segundo, 2016; Soto-Suarez et al., 2017). Here, we provide evidence that a polycistronic miRNA, miR166k-166h, plays a role in rice immunity. We show that accumulation of miR166k-166h transcripts in *miR166k-166h-Ac* mutant plants, and concomitant increase in mature miR166s derived from this precursor, results in enhanced resistance to infection by the rice blast fungus *M. oryzae*. Resistance to fungal infection in *miR166k-166h-Ac* plants is associated to a stronger induction of defence gene expression, at both the biotrophic (24- 48 hpi) and necrotrophic (72 hpi) stages of the infection. In wild-type plants, miR166k-166h accumulation increases not only during pathogen infection, but also in response to treatment with fungal elicitors, supporting that *MIR166k-166h* is a component of PTI. The up-regulation of miR166k-166h accumulation during infection and its relation to defence is also confirmed by the high expression in resistant rice cultivars. Whether *MIR166k-166h* plays a role in the rice response to infection by other pathogens remains to be determined.

Based on miRBase, the miR166 family comprises multiple members in monocotyledonous and dicotyledonous plants, which are transcribed independently*. In rice, loci* encoding monocistronic miR166k or miR166h are not identified, indicating that *MIR166k-166h* is the only source for production of mature miR166k and miR166h species. It is worth mentioning that *MIR166k-166h* can be found within the *O. sativa* genus (AA genome), present in *japonica* and *indica* subspecies, but it is also found in the genome of wild relatives of current cultivated rice varieties, that is, *O. rufipogon* and *O. nivara* (wild relatives of *O. sativa*) and *O. barthii* (wild relative of *O. glaberrima*, or African rice), further supporting conservation of the miR166k-166h polycistron in the *Oryza* genus (Baldrich et al. 2016). Furthermore, miR166 clusters have been identified in the genome of several plant species, such as *M. truncatula,* soybean and *Physcomitrella patens*, among others (Boualem et al. 2008; Zhang et al. 2009; Barik et al. 2014; Li et al. 2017) but, in most cases, the polycistronic nature of these miR166 clusters has not been demonstrated.

Evidence here presented support that *EIN2* and *ferredoxin-nitrite reductase* are novel target genes for miR166 species, particularly for the miR166k* and miR166h* sequences encoded by *MIR166k-166h*. Firstly, we show that there is an inverse correlation between miRNA levels (miR166k*, miR166h*) and accumulation of the corresponding target transcripts (*OsEIN2.1/OsEIN2.2* for miR166k*; *ferredoxin-nitrite reductase* for miR166h*) in *MIR166k-166h-Ac* mutant plants. Secondly, miR166s (miR166k*, miR166h*) and target genes *have* opposite expression patterns in response to fungal infection (up-regulation and down-regulation, respectively). Thirdly, miR166k*- and miR166h*-mediated cleavage events corresponding to *EIN2* and *ferredoxin-nitrite reductase* transcripts were identified by 5'-RACE analyses. Finally, a miR166-guided cleavage of *EIN2* transcripts was demonstrated in agro-infiltration experiments in *N. benthamiana* leaves. The observed reduction in *EIN2* transcripts is accompanied by a lower accumulation of EIN2 protein. From these results, it is concluded that *EIN2* and *ferredoxin-nitrite reductase* represent novel target genes of miR166.

Regarding the newly identified miR166 target genes, a function for *EIN2* as a mediator of ethylene-dependent defence responses is well established (Lorenzo *et al.*, 2003; Bailey *et al.*, 2009; Tsuda *et al.*, 2009; Helliwell *et al.*, 2016). For *ferredoxin-nitrite reductase* its possible role in plant immunity remains unknown. Only, recently was described that its expression is down-regulated during infection of a resistant chickpea cultivar with the fungal pathogen *Fusarium oxysporum*, but up-regulated during interaction with a susceptible cultivar (Upasani et al. 2017). *A*lthough conservation of miR166 and target genes suggest an important function of the miR166*/HD-ZIPIII* pair in the plant, it is also true that the existence of multiple miR166 family members might contribute to diversification and functional specialization of miR166 in plants. In line with this, miR166b has been reported to target *RDD1*, a non *HD-ZIP III* transcription factor (Iwamoto and Tagiri 2016). In *M. truncatula*, a miR166 polycistron containing tandem copies of miR166a has been shown to control root architecture and nodule development after infection by *Sinorhizobium meliloti* (Boualem et al. 2008). Presumably, miR166k-166h miRNAs would have evolved to mediate rice defence responses to pathogen infection.

When considering the mature miR166s encoded by the miR166k-166h precursor, we noticed that the miR166 species targeting *EIN2* or *ferredoxin-nitrite reductase* correspond to the miR166* sequences in monocistronic miR166s. Hence, the function of the miR166k-166h polycistron in rice immunity relies on the activity of miR166k* and/or miR166h* sequences, the two strands of the miR166/miR166* duplexes in the miR166k-166h precursor being functional. The best known example in the literature in which the two strands of the miRNA/miRNA* duplex are functional is the ath-miR393, in which miR393 guides cleavage of transcripts encoding auxin receptor genes (*TIR1*, *AFB2*, *AFB3*), whereas miR393* cleaves *MEMB12* transcripts encoding a SNARE protein involved in exocytosis of the Pathogenesis-Related PR1 protein (Zhang et al. 2011b). In *Medicago truncatula*, miR166e* and miR166h* have been shown to mediate transcript cleavage involved in arbuscular mycorrhizal symbiosis (Devers et al. 2011). The ability of miRNA precursors to give rise to two mature functional strands in the same miRNA/miRNA* duplex might represent an effective strategy to diversify miRNA function.

An intriguing aspect of this study was the finding that *EIN2* expression appears to be oppositely regulated in *miR166k-166h-Ac* plants. Whereas miR166k* down-regulates the expression of *EIN2.1* and *EIN2.2* (these *EIN2* genes being more closely related to one another than either to *EIN2.3* or *EIN2.4*), *EIN2.3* and *EIN2.4* are up-regulated in these plants. This observation raises the possibility of interconnecting networks controlling the expression of the *EIN2* gene family themselves, and that the abundance of a particular *EIN2* family member might affect the level of another *EIN2* family member. If so, the observed miR166-mediated down-regulation of *EIN2.1* and *EIN2.2* might be compensated by up-regulation of *EIN2.3* and *EIN2.4* in the *miR166k-166h-Ac* rice mutant. Further studies are required to understand the mechanisms by which *EIN2.3* and *EIN2.4* are up-regulated in the miR166k-166h mutant. Results here presented indicated that *MIR166k-166h* activation enhances defence gene expression, most probably, through modulation of *OsEIN2* expression. In this way, miR166k* would fine-tune *OsEIN2* expression, rather than completely turning *OsEIN2* expression off. If so, this would represent an additional layer of regulation of *OsEIN2* expression in rice which would help in maintaining appropriated OsEIN2 levels to allow optimal expression of defence responses with no negative impact on plant growth. We show that *MIR166k-166h* expression is itself regulated by treatment with the ethylene precursor ACC (**Fig. 7**).

In rice, basal resistance to *M. oryzae* has been reported to require ethylene biosynthesis during the biotrophic phase, and activation of ethylene signalling pathways (Helliwell et al. 2016b; Singh et al. 2004; Iwai et al. 2006; Helliwell et al. 2013; Yang et al. 2017). However, the mechanisms by which the pathogen induces ethylene biosynthesis remain unknown. Besides playing a role in plant responses to pathogen infection, ethylene is regarded as a phytohormone involved in regulation of plant growth and development. Excessive ethylene production under pathogen infection might negatively affect plant development. The host plant must then maintain a tight control of ethylene homeostasis to cope with pathogenic infections with no growth penalty. Along with this, negative feedback mechanisms for regulation of ethylene biosynthesis and signalling have been described (Vandenbussche et al. 2012). Such negative feedback mechanisms have been proposed to coordinate plant growth and ethylene/salinity responses (Tao et al. 2015) . The *M. oryzae*-induced production of ethylene, and subsequent activation of *MIR166k-166h* expression, would keep *OsEIN2* at appropriate levels which might be critical in *responses* to pathogen infection and developmental cues.

The *M.oryzae*-mediated ethylene accumulation has been shown to affect JA signalling (Yang et al 2017). Because ethylene is involved in the complex crosstalk of defence-signalling hormones (SA, ET, JA and ABA), the final outcome of the plant-pathogen interaction might well rely on the balance between hormone signalling pathways and on the stage of the *M. oryzae* infection process (biotrophic or necrotrophic) (Kankanala et al. 2007; Campos-Soriano et al. 2013; Wilson and Talbot 2009). Whether miR166k-166h activation has an impact on ethylene levels, and defence hormone networking, deserves further investigation. Literature also supports that ethylene might play antagonistic effects in controlling the rice defence response depending not only on the pathogen lifestyle but also on the type of pathogen. Whereas accumulation of ethylene appears to be required for resistance against *M. oryzae* (Iwai et al. 2006), repression of ethylene signalling has been shown to enhance resistance against the necrotrophic rice brown spot fungus *Cochliobolus miyabeanus* (Vleesschauwer et al. 2010). A major future challenge is to find out the molecular processes by which *MIR166k-166h* function is integrated in the complex regulatory mechanisms involved in fine-tuning adaptive responses to fungal infection.

Given the well-established roles of miR166 and its *HD ZIP III* target genes in controlling developmental processes in a broad range of plant species, an intriguing question is why *MIR166k-166h* activation does not affect normal growth in the mutant plants. A possible explanation is that there might be a matter of threshold in miR166k-166h level (and subsequent miR166-regulated *Oshox* transcripts) and, also that the mutant plants under heterozygosity do not accumulate sufficient levels of miR166k* species to alter normal developmental programs. Clearly, moderate levels of mature miR166s produced by the miR166k-166h polycistron would provide a means to mount a more successful defence response without no penalty on normal development due to excessive down-regulation of miR166 *HD ZIP III* target genes.

In conclusion, results here presented support that miR166k-166h is a positive regulator of rice immunity through regulation of *OsEIN2* (and possibly *ferredoxin-nitrite reductase*) expression. Although the functional significance of the organization of miRNAs as polycistrons is still an issue of debate, polycistronic transcription can provide fine-tune gene expression in related or unrelated biological processes. As an additional advantage, polycistronic transcription allows coordinated spatial and/or temporal expression of miRNAs (a single promoter can drive expression of multiple miRNAs). A better knowledge of miR166k-166h functioning in blast resistance will help in deciphering the functional consequences of polycistronic expression of miRNAs in plants. Since blast is one of the primary causes of rice losses worldwide, unraveling miR166k-166h-mediated mechanisms underlying blast resistance has the potential to ultimately help in designing novel strategies for crop protection.

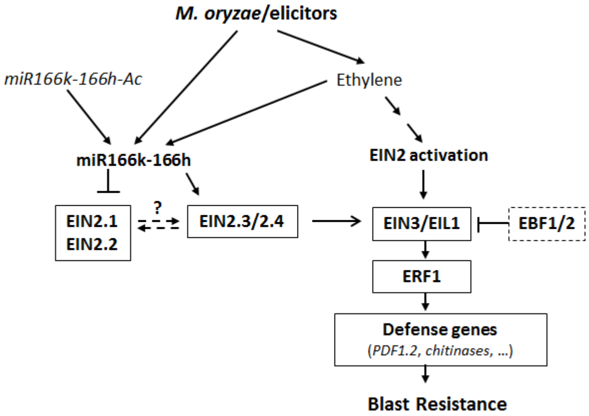

Figure 7. Schematic diagram of the role of miR166k-166h in ethylene signal transduction pathway during infection by the rice blast fungus *M. oryzae*. In the absence of ethylene, active ethylene receptors negatively regulate EIN2 through phosphorylation via the protein kinase CTR1 (CONSTITUTIVE TRIPLE-RESPONSE1) preventing the signal from transduction. Pathogen recognition triggers ethylene biosynthesis which is perceived by its receptors. Upon ethylene perception, the EIN2 phosphorylation is reduced and the carboxy-terminal fragment of EIN2 is cleaved and translocated to the nucleus for activation of EIN3/ELI1 and Ethylene Response Factors (ERFs) leading to activation of defence gene expression. Pathogen-induced ethylene production would also induce *MIR166k-166h* expression which would then act as a modulator of ethylene responses. A miR166k*-mediated cleavage of *OsEIN2* transcripts might allow to coordinate ethylene-dependent defence responses and plant growth.

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References

Abiri R, Azmi N, Mahmood S, et al. 2017. Role of ethylene and the APETALA 2/Ethylene Response Factor superfamily in rice under various abiotic and biotic stress conditions. Environmental and Experimental Botany 134, 33–44.

Agalou A, Purwantomo S, Övernäs E, et al. 2008. A genome-wide survey of HD-Zip genes in rice and analysis of drought-responsive family members. Plant Molecular Biology 66, 87–103.

Agrawal GK, Rakwal R, Jwa NS, Agrawal VP. 2001. Signalling molecules and blast pathogen attack activates rice OsPR1a and OsPR1b genes: A model illustrating components participating during defence/stress response. Plant Physiology and Biochemistry 39, 1095–1103.

Akdogan G, Tufekci ED, Uranbey S, Unver T. 2016. miRNA-based drought regulation in wheat. Functional and Integrative Genomics 16, 221–233.

Allen E, Xie Z, Gustafson AM, Sung G, Spatafora JW, Carrington JC. 2004. Evolution of microRNA genes by inverted duplication of target gene sequences in Arabidopsis thaliana. Nature Genetics 36, 1282–1290.

Bailey TA, Zhou X, Chen J, Yang Y. 2009. Role of Ethylene, Abscisic Acid and MAP Kinase Pathways in Rice Blast Resistance. G.-L. Wang, B. Valent (eds.), Advances in Genetics, Genomics and Control of Rice Blast Disease, DOI 10.1007/978-1-4020-9500-9 19, 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 Biology 12, 847–863.

Baldrich P, Hsing YC, Segundo BS. 2016. Genome-Wide Analysis of Polycistronic MicroRNAs in Cultivated and Wild Rice. Genome Biology Evolution 8, 1104–1114.

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

Bari R, Jones JDG. 2009. Role of plant hormones in plant defence responses. Plant Molecular Biology 69, 473–488.

Barik S, Sarkardas S, Singh A, Gautam V, Kumar P, Majee M, Sarkar AK. 2014. Genomics Phylogenetic analysis reveals conservation and diversi fi cation of micro RNA166 genes among diverse plant species. Genomics 103, 114–121.

Baumberger N, Baulcombe DC. 2005. Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. PNAS 102, 11928-11933

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.

Boualem A, Laporte P, Jovanovic M, Laffont C, Plet J, Combier JP, Niebel A, Crespi M, Frugier F. 2008. MicroRNA166 controls root and nodule development in Medicago truncatula. Plant Journal 54, 876–887.

Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. Science 320, 1185–1190.

Calviño M, Messing J. 2013. Discovery of MicroRNA169 Gene Copies in Genomes of Flowering Plants through Positional Informationnd messing 2013. Genome Biology and Evolution 5, 402–417.

Campo S, Peris-Peris C, Siré C, Moreno AB, Donaire L, Zytnicki M, Notredame C, Llave C, San Segundo B. 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, San Segundo B. 2012. The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defence-related genes in rice leaves and confers resistance to pathogen infection. Molecular Plant Pathology 13, 579–592.

Campos-Soriano L, Valè G, Lupotto E, San Segundo B. 2013. Investigation of rice blast development in susceptible and resistant rice cultivars using a gfp- expressing Magnaporthe oryzae isolate. Plant pathology 9, 1030–1037.

Casacuberta JM, Raventós D, Puigdoménech P, San Segundo B. 1992. Expression of the gene encoding the PR-like protein PRms in germinating maize embryos. Molecular & General Genetics 234, 97–104.

Chen X. 2009. Small RNAs and Their Roles in Plant Development. Annu Rev 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. The Plant Cell 18, 412–421.

Chuck G, Meeley R, Irish E, Sakai H, Hake S. 2007. The maize tasselseed4 microRNA controls sex determination and meristem cell fate by targeting Tasselseed6/indeterminate spikelet1. Nature genetics 39, 1517–1521.

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. Frontiers in Plant Science 4, 1–12.

Devers EA, Branscheid A, May P, Krajinski F. 2011. Stars and Symbiosis: MicroRNA- and MicroRNA * -Mediated Transcript Cleavage Involved in Arbuscular Mycorrhizal Symbiosis. Plant Physiology 156, 1990–2010.

Ding J, Jia J, Yang L, Wen H, Zhang C, Liu W, Zhang D. 2004. Validation of a Rice Specific Gene, Sucrose Phosphate Synthase , Used as the Endogenous Reference Gene for Qualitative and Real-Time Quantitative PCR detection of transgenes. Journal of Agricultural and Food Chemistry 53, 3372–3377.

Ding Y, Ye Y, Jiang Z, Wang Y, Zhu C. 2016. MicroRNA390 Is Involved in Cadmium Tolerance and Accumulation in Rice. Frontiers in Plant Science 7, 1–10.

Emery JF, Floyd SK, Alvarez J, Eshed Y, Hawker NP, Izhaki A, Baum SF, Bowman JL. 2003. Radial Patterning of Arabidopsis Shoots by Class III HD-ZIP and KANADI Genes. Current Biology 13, 1768–1774.

Fahlgren N, Howell MD, Kasschau KD, Chapman EJ, Sullivan CM, Cumbie JS, Givan SA. 2007. High-Throughput Sequencing of Arabidopsis microRNAs: Evidence for Frequent Birth and Death of MIRNA Genes. PLoS ONE 219, 1–14.

Fei Q, Zhang Y, Xia R, Meyers BC. 2016. Small RNAs Add Zing to the Zig-Zag-Zig Model of Plant Defenses. MPMI 29, 165–9.

Felippes FF, Schneeberger K, Dezulian T, Huson DH, Weigel D. 2008. Evolution of Arabidopsis thaliana microRNAs from random sequences. RNA 14, 2455–2459.

Gao S, Yang L, Zeng HQ, Zhou ZS, Yang ZM, Li H, Sun D, Xie F, Zhang B. 2016. A cotton miRNA is involved in regulation of plant response to salt stress. Scientific Reports 6, 19736.

Glazebrook J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual Review of Phytopathology 43, 205–27.

Hamza NB, Sharma N, Tripathi A, Sanan-Mishra N. 2016. MicroRNA expression profiles in response to drought stress in Sorghum bicolor. Gene Expression Patterns 20, 88–98.

Helliwell EE, Wang Q, Yang Y. 2013. Transgenic rice with inducible ethylene production exhibits broad-spectrum disease resistance to the fungal pathogens Magnaporthe oryzae and Rhizoctonia solani. Plant Biotechnology Journal 11, 33–42.

Helliwell EE, Wang Q, Yang Y. 2016. Ethylene biosynthesis and signaling is required for rice immune response and basal resistance against Magnaporthe oryzae infection. MPMI, http://dx.doi.org/10.1094/MPMI-06-16-0121-R.

Hsing Y, Lu C, Fan M, et al. 2007. A rice gene activation / knockout mutant resource for high throughput functional genomics. Plan Molecular Biology 63, 351–364.

Huang J, Yang M, Zhang X. 2016. The function of small RNAs in plant biotic stress response. Journal of Integrative Plant Biology 58, 312–327.

Itoh J-I, Hibara K-I, Sato Y, Nagato Y. 2008. Developmental role and auxin responsiveness of Class III homeodomain leucine zipper gene family members in rice. Plant Physiology 147, 1960–75.

Iwai T, Miyasaka A, Seo S, Ohashi Y. 2006. Contribution of Ethylene Biosynthesis for Resistance to Blast Fungus Infection in Young Rice Plants. Plant Physiology 142, 1202–1215.

Iwamoto M, Tagiri A. 2016. MicroRNA-targeted transcription factor gene RDD1 promotes nutrient ion uptake and accumulation in rice. The Plant Journal 85, 466–477.

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

Jones-Rhoades MW, Bartel DP. 2004. Computational Identification of Plant MicroRNAs and Their Targets, Including a Stress-Induced miRNA. Molecular Cell 14, 787–799.

Jones-Rhoades MW, Bartel DP, Bartel B. 2006. MicroRNAS and their regulatory roles in plants. Annual Review of Plant Biology 57, 19–53.

Ju C, Yoon GM, Shemansky JM, et al. 2012. CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 109, 19486–91.

Jun SH, Han MJ, Lee S, Seo YS, Kim WT, An G. 2004. OsEIN2 is a positive component in ethylene signaling in rice. Plant and Cell Physiology 45, 281–289.

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.

Kitajima S, Sato F. 1999. Review Pathogenesis-Related and Protein Proteins: Molecular Mechanisms of Gene Expression and Protein Function. Journal of Biochemistry 125, 1–8.

Kitazumi A, Kawahara Y, Onda TS, De Koeyer D, de los Reyes BG, Cloutier S. 2015. Implications of miR166 and miR159 induction to the basal response mechanisms of an andigena potato (Solanum tuberosum subsp. andigena) to salinity stress, predicted from network models in Arabidopsis. Genome 58, 13–24.

Kuan T, Zhai Y, Ma W. 2016. Small RNAs regulate plant responses to filamentous pathogens. Seminars in Cell and Developmental Biology 56, 190–200.

Kurihara Y, Watanabe Y. 2004. Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. Proceedings of the National Academy of Sciences of the United States of America 101, 12753–12758.

Li Y, Lu Y-G, Shi Y, Wu L, Xu Y-J, Huang F, Guo X-Y, Zhang Y, Zhao J-Q, Zhang H-Y, Xu P-Z, Zhou J-M, Wu X-J, Wang P-R, Wang W-M. 2014. Multiple rice microRNAs are involved in immunity against the blast fungus Magnaporthe oryzae. Plant Physiology 164, 1077–92.

Li Z-Y, Xia J, Chen Z, Yu Y, Li Q-F, Zhang Y-C, Zhang J-P, Wang C-Y, Zhu X-Y, Zhang W, Chen Y-Q. 2016. Large-scale rewiring of innate immunity circuitry and microRNA regulation during initial rice blast infection. Scientific Reports 6, 25493.

Li X, Xie X, Li J, Cui Y, Hou Y, Zhai L, Wang X, Fu Y, Liu R, Bian S. 2017. Conservation and diversification of the miR166 family in soybean and potential roles of newly identified miR166s. BMC Plant Biology 17, 32.

Llave C, Franco-Zorrilla JM, Solano R, Barajas D. 2011. Target Validation of Plant microRNAs. Methods in Molecular Biology 732, 187–208.

Llave C, Kasschau KD, Rector MA, Carrington JC. 2002. Endogenous and Silencing-Associated Small RNAs in Plants. The Plant Cell 14, 1605–1619.

Lorenzo O, Piqueras R, Sánchez-serrano JJ, Solano R. 2003. ETHYLENE RESPONSE FACTOR1 Integrates Signals from Ethylene and Jasmonate Pathways in Plant Defense. Plant Cell 15, 165–178.

Ma B, He SJ, Duan KX, et al. 2013. Identification of rice ethylene-response mutants and characterization of MHZ7/OsEIN2 in distinct ethylene response and yield trait regulation. Molecular Plant 6, 1830–1848.

Mallory AC, Reinhart BJ, Jones-Rhoades MW, Zamore PD, Kathryn M, Bartel DP. 2004. MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region. The EMBO Journal 23, 3356–3364.

Merchan F, Boualem A, Crespi M, Frugier F. 2009. Plant polycistronic precursors containing non-homologous microRNAs target transcripts encoding functionally related proteins. Genome Biology 10, R136.

Midoh N, Iwata M. 1996. Cloning and characterization of a probenazole-inducible gene for an intracellular pathogenesis-related protein in rice. Plant & Cell Physiology 37, 9–18.

Nagasaki H, Itoh J, Hayashi K, Hibara K, Satoh-nagasawa N, Nosaka M. 2007. The small interfering RNA production pathway is required for shoot meristem initiation in rice. PNAS 104, 1–5.

Nozawa M, Miura S, Nei M. 2012. Origins and evolution of microRNA genes in plant species. Genome Biology and Evolution 4, 230–239.

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

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. Phytopathology 92, 870–876.

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

Rakwal R, Yang G, Komatsu S. 2004. Chitinase induced by jasmonic acid, methyl jasmonate, ethylene and protein phosphatase inhibitors in rice. Molecular Biology Reports 31, 113–119.

Rogers K, Chen X. 2013. Biogenesis, turnover, and mode of action of plant microRNAs. The Plant Cell 25, 2383–99.

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

Schommer C, Bresso EG, Spinelli S V, Palatnik JF. 2012. MicroRNAs in Plant Development and Stress Responses. Signaling and Comunications in Plants 15, 29–48.

Schwach F, Vaistij FE, Jones L, Baulcombe DC. 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 Physiology 138, 1842–1852.

Shivaprasad P V., Chen H-M, Patel K, Bond DM, Santos B a. CM, Baulcombe DC. 2012. A MicroRNA Superfamily Regulates Nucleotide Binding Site-Leucine-Rich Repeats and Other mRNAs. The Plant Cell 24, 859–874.

Singh MP, Lee FN, Counce PA, Gibbons JH, Barr H, Pyricularia S. 2004. Mediation of Partial Resistance to Rice Blast Through Anaerobic Induction of Ethylene. Phytopathology 94, 819–825.

Solano R, Ecker JR. 1998. Ethylene gas: perception, signaling and response. Current Opinion in Plant Biology 1, 393-8.

Soto-Suarez 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 7, 1–14.

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

Sunkar R, Chinnusamy V, Zhu J, Zhu J. 2007. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. Trends in Plant Science 12, 301–309.

Tacconi G, Baldassarre V, Lanzanova C, Cavigiolo S, Urso S, Lupotto E, Vale G. 2010. Polymorphism analysis of genomic regions associated with broad-spectrum effective blast resistance genes for marker development in rice. Molecular Breeding 26, 595–617.

Tang X, Bian S, Tang M, Lu Q, Li S, Liu X, Cui Y. 2012. MicroRNA – Mediated Repression of the Seed Maturation Program during Vegetative Development in Arabidopsis. Plos GENETICS 8, 20–22.

Tao J, Chen H, Ma B, Zhang W, Chen S, Zhang J. 2015. The Role of Ethylene in Plants Under Salinity Stress. Frontiers in Plant Science 6, 1–12.

Thiel T, Graner A, Waugh R, Grosse I, Close TJ, Stein N. 2009. Evidence and evolutionary analysis of ancient whole-genome duplication in barley predating the divergence from rice. BMC Evolutionary Biology 9, 1–18.

Tsuda K, Sato M, Stoddard T, Glazebrook J, Katagiri F. 2009. Network Properties of Robust Immunity in Plants. Plos GENETICS 5, e1000772.

Upasani ML, Limaye BM, Gurjar GS, Kasibhatla SM. 2017. Interaction transcriptome reveals differential modulation of plant defense strategies. Scientific Reports 7, 1–12.

Vandenbussche F, Vaseva I, Vissenberg K, Straeten D Van Der. 2012. Tansley review Ethylene in vegetative development: a tale with a riddle. New Phytologist 194, 895–909.

Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP. 2007. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods 3, 12.

Vleesschauwer D De, Yang Y, Cruz CV, Höfte M. 2010. Abscisic Acid-Induced Resistance against the Brown Spot Pathogen Cochliobolus miyabeanus in Rice Involves MAP Kinase-Mediated Repression of Ethylene Signaling. Plant Physiology 152, 2036–2052.

Voinnet O. 2009. Origin, Biogenesis, and Activity of Plant MicroRNAs. Cell 136, 669–687.

Wang N, Xiao B, Xiong L. 2011. Identification of a cluster of PR4-like genes involved in stress responses in rice. Journal of Plant Physiology 168, 2212–2224.

Weiberg A, Wang M, Bellinger M, Jin H. 2014. Small RNAs: A New Paradigm in Plant-Microbe Interactions. Annual Review of Phytopathology 52, 495–516.

Wilson RA, Talbot NJ. 2009. Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. Nature Reviews 7, 185–195.

Xu S, Liu N, Mao W, Hu Q, Wang G, Gong Y. 2016. Identification of chilling-responsive microRNAs and their targets in vegetable soybean (Glycine max L.). Scientific Reports 6, 26619.

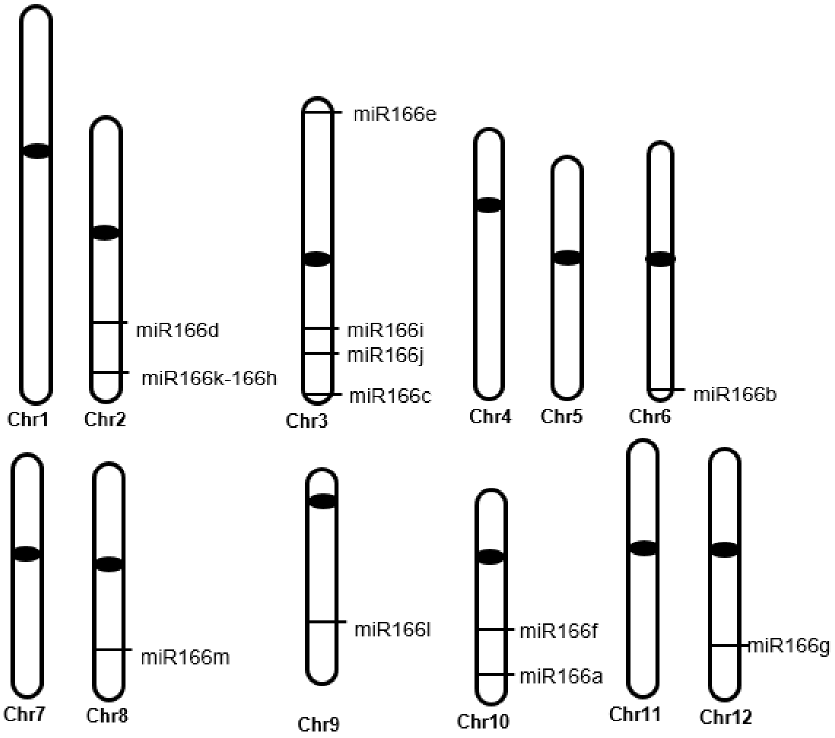
Yang Y, Ahammed GJ, Wu C, Fan S, Zhou Y. 2015. Crosstalk among Jasmonate, Salicylate and Ethylene Signaling Pathways in Plant Disease and Immune Responses. Current Protein and Peptide Science 16, 450–461.

Yang C, Li W, Cao J, Meng F, Yu Y, Huang J, Jiang L, Liu M. 2017. Activation of ethylene signaling pathways enhances disease resistance by regulating ROS and phytoalexin production in rice. The Plant Journal 89, 338–353.

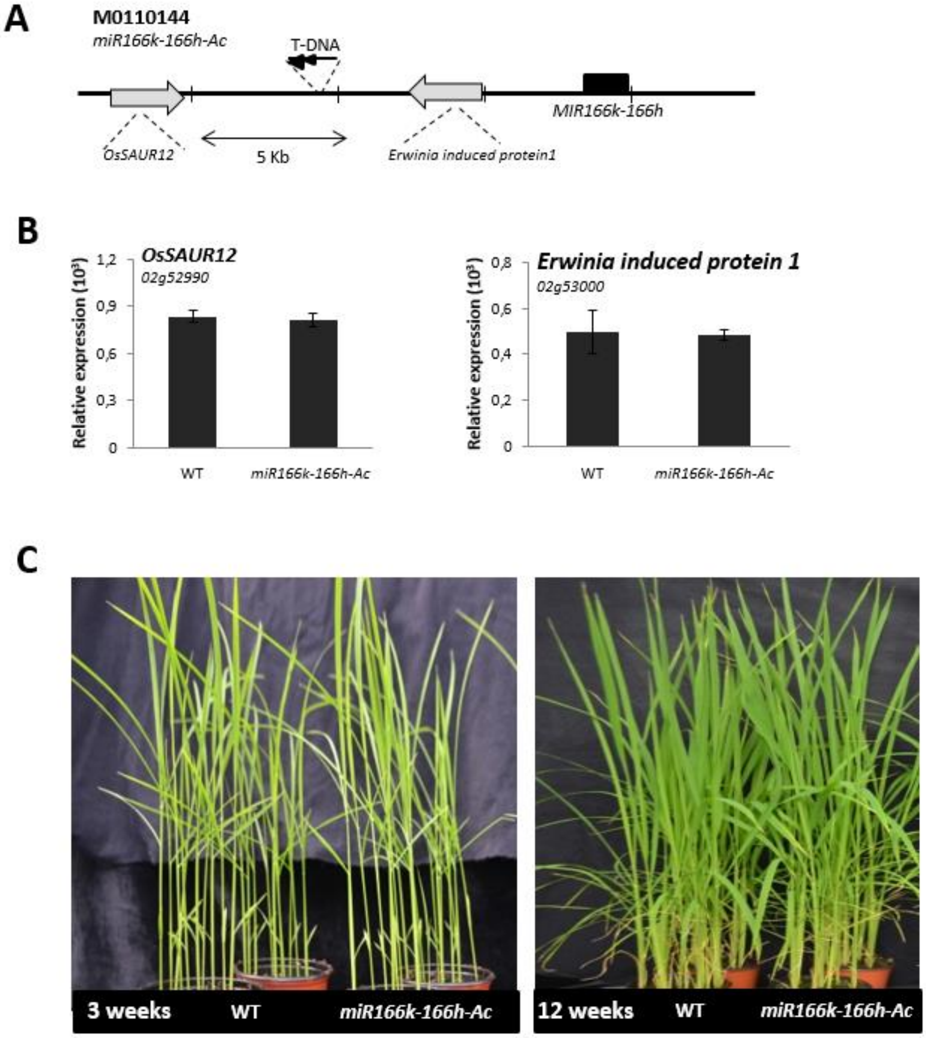
Zhang L, Chia J, Kumari S, Stein JC, Liu Z, Narechania A, Maher CA, Guill K, Mcmullen MD, Ware D. 2009. A Genome-Wide Characterization of MicroRNA Genes in Maize. Plos GENETICS 5, e1000716.

Zhang X, Zhao H, Gao S, Wang W-C, Katiyar-Agarwal S, Huang H-D, Raikhel N, Jin H. 2011. Arabidopsis Argonaute 2 regulates innate immunity via miRNA393*-mediated silencing of a Golgi-localized SNARE gene MEMB12. Molecular Cell 42, 356–366.

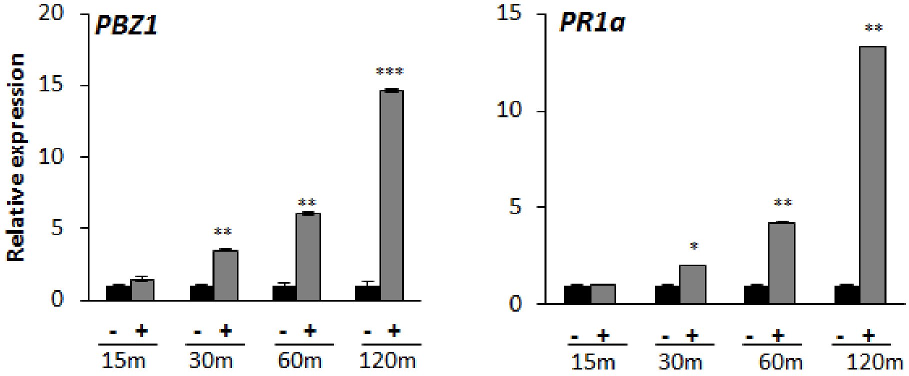
Supplemental Material



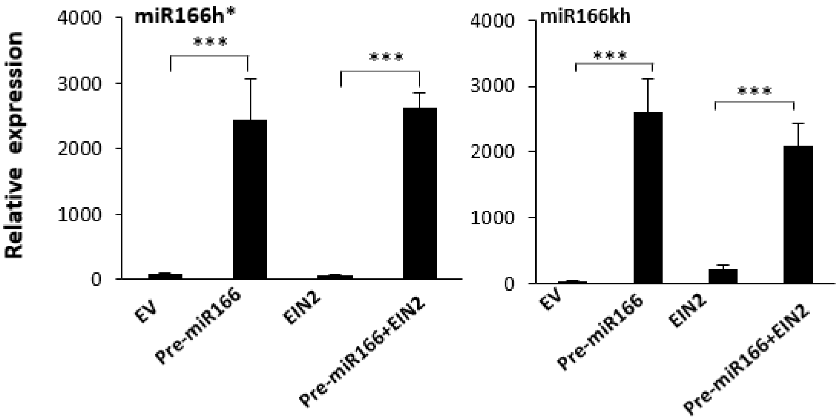
Supplemental Figure 1. Chromosomal location of monocistronic miR166s and polycistronic miR166k-166h in rice. The centromere in each chromosome is indicated by a black circle.



Supplemental Figure 2. Characterization of the *miR166k-166h-Ac* mutant from the TRIM collection. A. Schematic representation of the T-DNA in the *miR166k-166h-Ac* mutant. B. Expression of genes flanking the T-DNA was determined by RT-qPCR. Histograms show the mean ± SD, ANOVA test). C. Developmental phenotype of wild-type and *miR166k-166h-Ac* plants at 3 and 12 weeks.



Supplemental Figure 3. Expression of *PBZ* and *PR1a* in wild-type plants in response to treatment with *M. oryzae* elicitors. The accumulation of *PBZ* and *PR1a* transcripts was determined at the indicated times of elicitor treatment by RT-qPCR. Asterisks denote statistically significant differences (***, $P \leq 0.001$; **, $P \leq 0.01$, *, $P \leq 0.05$, ANOVA test; elicitor-treated *vs* mock-treated).

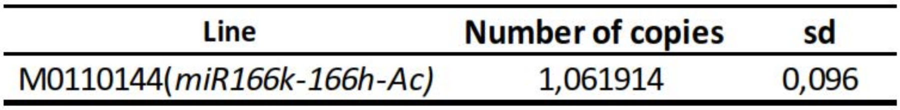


Supplemental Figure 4. Agroinfiltration assays in *N. benthamiana* leaves. Constructs harboring either the miR166k-166h precursor, the *EIN2-GFP* or the combination were assayed. The empty vector (EV) was used as control. Expression analyses were performed at 2 days after agroinfiltration. The accumulation of miR166k-166h precursor transcripts was determined by RT-qPCR, whereas that of miR166h* and miR166 mature sequences was assayed by ST-RT-PCR. Histograms show the mean ± SD (***, $P \leq 0.001$; ANOVA test).

Supplementary Table 1. Oligonucleotides used in this study.

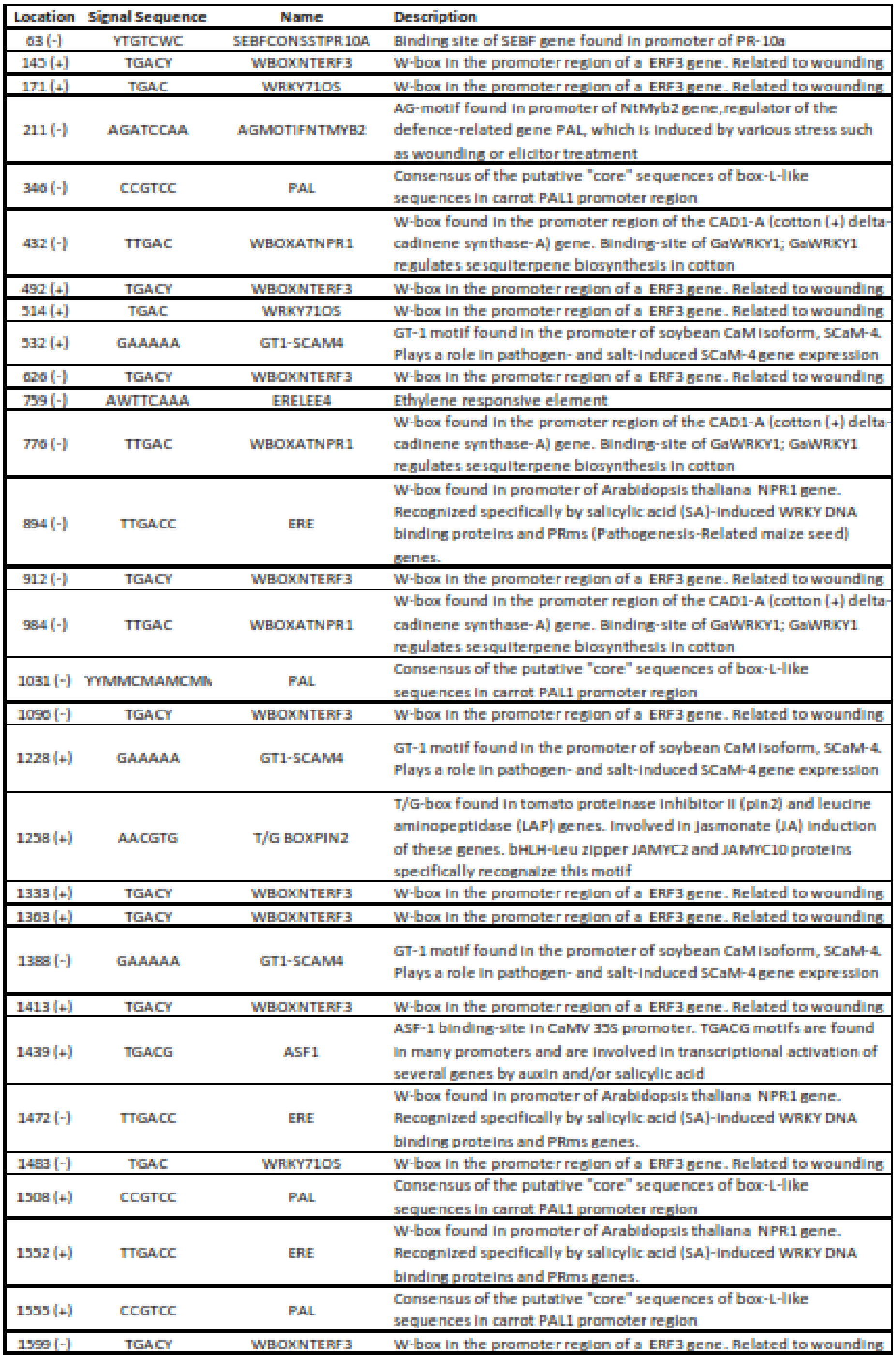
Oligo ID	***Accesion number***		**Sequence (5'-3')**
For expression analysis			
Cyclophilin 2	*LOC_Os02g02890*	*Frw*	GTGGTGTTAGTCTTTTTATGAGTTCGT
		Rev	ACCAAACCATGGGCGATCT
pre-miR166k-166h		*Frw*	CCTCGAGCCAGACAACAAAC
		Rev	GGTGGCTTGTGGGGAATGTTGGCTGG
OsSAUR12	*LOC_Os02g52990*	*Frw*	GGGGGAGGAGAAGAAGG
		Rev	CCGCCAGCCACCCTTTCT
Erwinia induced protein 1	*LOC_Os02g53000*	*Frw*	ATGGCGGGGGTGTGTGG
		Rev	ACGACGACACCATCAC
PR1a	*LOC_Os07g03710*	*Frw*	CTGCAAGCTGGAGCACTCG
		Rev	AAGATGTTCTCGCCGTACTTCC
PBZ	*LOC_Os12g36830*	*Frw*	GCGATGGCTCCTGTGTGG
		Rev	CTCCGGCGACAGTGAGCT
PDF1.2	*LOC_OsOs02g12060*	*Frw*	GACGACGAGGAGGAGGACGA
		Rev	ATGGAGCCTTCAGGCAAGCT
PR3(CHIT8)	*LOC_Os06g51060*	*Frw*	TCCCACCGCTTCTTAACATCA
		Rev	ATGGTGGCAATGCAAAGCAAA
PR3(CHIT14)	*LOC_Os10g39680*	*Frw*	CTTCTACACGTACGACGCCTTCATCGCC
		Rev	AGGTGCCGAACGCCGGGAA
PR4(WIP5)	*LOC_Os11g37970*	*Frw*	CTGCTGCACAAGAAGCATCC
		Rev	CCCAGTTGTTCTGTTGTGGG
EIN3	*LOC_Os03g20780*	*Frw*	ATGGGAGGTGGTCTGGTGATGGA
		Rev	CCACGAAGTTGTGCACGCC
EIL1	*LOC_Os03g20790*	*Frw*	TACAGATTGAAGAAGGCTTTTTT
		Rev	AACACACCTCCGTTGCTGCCGCC
ERF1	*LOC_Os04g46220*	*Frw*	GAGTCGTCCTTCTCCTCCTC
		Rev	CCTCTCTTTCTCCGTTTCG
EBF2	*LOC_Os06g40360*	*Frw*	TGGTGGTGTTCTTGTGATGG
		Rev	TGACGCCGCCGAACAGAGCC
hox9	*LOC_Os10g33960*	*Frw*	GGGACTGCTGTTGATTGGGTG
		Rev	GCCACAATACCAAACGAAT
hox10	*LOC_Os03g01890*	*Frw*	ATTGATTGGGTCCAGATGCC
		Rev	GCCACAATACCAACCGAAT
hox29	*LOC_Os01g10320*	*Frw*	GTGATCACAGGATGGGGAAGGC
		Rev	CTCTGGTGAGCTTTTGGCTCAGA
hox32	*LOC_Os03g43930*	*Frw*	GTCGATTGGGTGCAAATGGT
		Rev	TGTGCGAAACAGCGATGAT
hox33	*LOC_Os12g41860*	*Frw*	TGCTGTCGAATGGGTGCAAATGG
		Rev	GAAACAGCGATGATTCCAATGGAAT
EIN2.1	*LOC_Os07g06130*	*Frw*	TGTTGGTGACTGAGAAGCCAT
		Rev	ATGAGAAGTCCCTCTTGGGAA
EIN2.2	*LOC_Os03g49400*	*Frw*	AACGGCGTGTGTTTGGTTAAT
		Rev	CGCGATACTACGCCTGGTC
EIN2.3	*LOC_Os07g06300*	*Frw*	TCTTTCTAGCTCCGCCATGC
EIN2.4	*LOC_Os07g06190*	*Rev*	ATGCATGTGTCAACCGAAGC
ferredoxin nitrite reductase	*LOC_Os02g52730*	*Frw*	GCGAGCGTGATCGAGGCGTA
		Rev	CAGTTCTGGCGGGTTGTCA
miR166k-5p		*Frw*	GGCGGGGTTTGTTGTCTGG
		SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCTCGA
miR166h-5p		*Frw*	GGCGGGGAATGTTGGCTGG
		SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCTCGA
miR166k-3p		*Frw*	GGCGGTCGGACCAGGCTTC
		SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGAT
miR166h-3p		*Frw*	GGCGGTCGGACCAGGCTTC
		SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGGAA
For fungal DNA quantification			
M. oryzae 28S		*Frw*	TACGAGAGGAACCGCTCATTCAGATAATTA
		Rev	TCAGCAGATCGTAACGATAAAGCTACTC
For 5' RACE			
EIN2		*Rev*	ACCTATCCGGGATGTTGCTGCGATCTGT
		Rev-Nested	TCCCATGGTAATCAAAGAGATGCCCCCAGAA
ferredoxin nitrite reductase		*Rev*	AGCGCCGGCAAGATCGTCGAGG
		Rev-Nested	GGAGAGGGAGGAGAACGAGGAGTAG
Hox32		*Rev*	CCCCCCTCACATGGGCGGATCAAATAGCCGC
		Rev-Nested	CCGCCAAAGTTGTCGGTGCATAAGTCTGCA
5' RACE kit Fw primers		*Fw*	CGACTGGAGCACGAGGACACTGA
		Fw-Nested	GGACACTGACATGGACTGAAGGAGTA
For miR166k-166h overexpression			
miR166k-166h		*Frw*	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGTGGCTTGTGGGGAATGTT
		Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTAAAGGGATTGAAGCCTGGT
For *Nicothiana benthamiana* agroinfiltration analysis			
N. benthamiana ubiquitin		*Frw*	TCCAGGACAAGGAGGGTATCC
		Rev	TAGTCAGCCAAGGTCCTTCCAT
miR166k-5p probe			CCTCGAGCCAGACAACAAACC
U6			TCATCCTTGCGCAGGGGCCA
For polycistronic miRNA genotyping			
M0110144		*Frw*	CCGGGCAGTGGATTGCGTTGT
		Rev	CAAATACTACCAAATATAAGTCACCGG
T-DNA LB		*Frw*	ACTCATGGCGATCTCTTACC
For transgene copy insertions			
Sucrose phosphate synthase	*LOC_Os02g09170*	*Frw*	TTGCGCCTGAACGGATAT
		Rev	CATCCCGAAAAGATCAACCG
Hygromycin		*Frw*	CTATTTCTTTGCCCTCGGACGA
		Rev	CTTCTACACAGCCATCGGTCC

Supplementary Table 2. T-DNA copy number in miR166k-166h plants. The T-DNA copy number was determined by qPCR using the *sucrose phosphate synthase* (*SPS*) gene as the endogenous reference gene (Iu and Hang 2004).



Line	Number of copies	sd
M0110144(*miR166k-166h-Ac)*	1,061914	0,096

Supplementary Table 3. *Cis*-motifs identified in the *MIR166k-166h* promoter.



Location	Signal Sequence	Name	Description
63 (-)	YTGTCWC	SEBFCONSSTPR10A	Binding site of SEBF gene found in promoter of PR-10a
145 (+)	TGACY	WBOXNTERF3	W-box in the promoter region of a ERF3 gene. Related to wounding
171 (+)	TGAC	WRKY71OS	W-box in the promoter region of a ERF3 gene. Related to wounding
211 (-)	AGATCCAA	AGMOTIFNTMYB2	AG-motif found in promoter of NtMyb2 gene,regulator of the defence-related gene PAL, which is induced by various stress such as wounding or elicitor treatment
346 (-)	CCGTCC	PAL	Consensus of the putative "core" sequences of box-L-like sequences in carrot PAL1 promoter region
432 (-)	TTGAC	WBOXATNPR1	W-box found in the promoter region of the CAD1-A (cotton (+) delta-cadinene synthase-A) gene. Binding-site of GaWRKY1; GaWRKY1 regulates sesquiterpene biosynthesis in cotton
492 (+)	TGACY	WBOXNTERF3	W-box in the promoter region of a ERF3 gene. Related to wounding
514 (+)	TGAC	WRKY71OS	W-box in the promoter region of a ERF3 gene. Related to wounding
532 (+)	GAAAAA	GT1-SCAM4	GT-1 motif found in the promoter of soybean CaM isoform, SCaM-4. Plays a role in pathogen- and salt-induced SCaM-4 gene expression
626 (-)	TGACY	WBOXNTERF3	W-box in the promoter region of a ERF3 gene. Related to wounding
759 (-)	AWTTCAAA	ERELEE4	Ethylene responsive element
776 (-)	TTGAC	WBOXATNPR1	W-box found in the promoter region of the CAD1-A (cotton (+) delta-cadinene synthase-A) gene. Binding-site of GaWRKY1; GaWRKY1 regulates sesquiterpene biosynthesis in cotton
894 (-)	TTGACC	ERE	W-box found in promoter of Arabidopsis thaliana NPR1 gene. Recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins and PRms (Pathogenesis-Related maize seed) genes.
912 (-)	TGACY	WBOXNTERF3	W-box in the promoter region of a ERF3 gene. Related to wounding
984 (-)	TTGAC	WBOXATNPR1	W-box found in the promoter region of the CAD1-A (cotton (+) delta-cadinene synthase-A) gene. Binding-site of GaWRKY1; GaWRKY1 regulates sesquiterpene biosynthesis in cotton
1031 (-)	YYMMCMAMCMN	PAL	Consensus of the putative "core" sequences of box-L-like sequences in carrot PAL1 promoter region
1096 (-)	TGACY	WBOXNTERF3	W-box in the promoter region of a ERF3 gene. Related to wounding
1228 (+)	GAAAAA	GT1-SCAM4	GT-1 motif found in the promoter of soybean CaM isoform, SCaM-4. Plays a role in pathogen- and salt-induced SCaM-4 gene expression
1258 (+)	AACGTG	T/G BOXPIN2	T/G-box found in tomato proteinase inhibitor II (pin2) and leucine aminopeptidase (LAP) genes. Involved in jasmonate (JA) induction of these genes. bHLH-Leu zipper JAMYC2 and JAMYC10 proteins specifically recognaize this motif
1333 (+)	TGACY	WBOXNTERF3	W-box in the promoter region of a ERF3 gene. Related to wounding
1363 (+)	TGACY	WBOXNTERF3	W-box in the promoter region of a ERF3 gene. Related to wounding
1388 (-)	GAAAAA	GT1-SCAM4	GT-1 motif found in the promoter of soybean CaM isoform, SCaM-4. Plays a role in pathogen- and salt-induced SCaM-4 gene expression
1413 (+)	TGACY	WBOXNTERF3	W-box in the promoter region of a ERF3 gene. Related to wounding
1439 (+)	TGACG	ASF1	ASF-1 binding-site in CaMV 35S promoter. TGACG motifs are found in many promoters and are involved in transcriptional activation of several genes by auxin and/or salicylic acid
1472 (-)	TTGACC	ERE	W-box found in promoter of Arabidopsis thaliana NPR1 gene. Recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins and PRms genes.
1483 (-)	TGAC	WRKY71OS	W-box in the promoter region of a ERF3 gene. Related to wounding
1508 (+)	CCGTCC	PAL	Consensus of the putative "core" sequences of box-L-like sequences in carrot PAL1 promoter region
1552 (+)	TTGACC	ERE	W-box found in promoter of Arabidopsis thaliana NPR1 gene. Recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins and PRms genes.
1555 (+)	CCGTCC	PAL	Consensus of the putative "core" sequences of box-L-like sequences in carrot PAL1 promoter region
1599 (-)	TGACY	WBOXNTERF3	W-box in the promoter region of a ERF3 gene. Related to wounding

Chapter II

OsDCL1a activation compromises disease resistance in rice

Abstract

MicroRNAs (miRNAs) are small non-coding RNAs acting as post-transcriptional regulators of gene expression through sequence-specific cleavage or translational repression of target transcripts in eukaryotes. They are transcribed as long single-stranded RNA precursors with unique stem-loop structures that are processed by a DICER-Like (DCL) ribonuclease, typically DCL1, to produce mature miRNA sequences. Plant miRNAs have been implicated in developmental processes and adaptation to the environment. Here, we investigated the contribution of *OsDCL1* to rice immunity. We show that activation-tagged *dcl1a* mutants (*dcl1a-Ac*) show enhanced susceptibility of infection by fungal pathogens, these plants being significantly compromised in pathogen-induced defense gene expression. No effect on plant growth and development was observed in *dcl1a-Ac* plants in the absence of pathogen challenge. To analyse the impact of *OsDCL1a* activation on the host transcriptome, we compared mRNA and miRNA transcriptomes of *dcl1a-Ac* and wild-type plants. In addition to defense-related genes, *OsDCL1a* activation results in misregulation of genes involved in detoxification of reactive oxygen species (ROS). Consistently, *DCL1a-Ac* plants are more sensitive to oxidative stress caused by methyl viologen treatment, and also accumulate high levels of $O_2^{\bullet-}$ in their leaves. Notably, transcriptome analysis revealed down-regulation of genes involved in the biosynthesis of diterpenoid phytoalexins in *dcl1a-Ac* plants compared to wild-type plants, the expression of these genes being also weakly induced during pathogen infection. Furthermore, our results indicate that *OsDCL1a* activation results in important alterations in the rice miRNAome, including both up-regulation and down-regulation of miRNAs. Our findings support a role of *OsDCL1a* in regulating rice immunity through an effect on ROS homeostasis and phytoalexin biosynthesis.

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional regulators of gene expression through sequence-specific cleavage or translational repression of target transcripts in eukaryotes (Llave et al. 2002; Brodersen et al. 2008). *MIRNA* genes are transcribed by RNA polymerase II into long precursor transcripts with unique stem-loop structures (pri-miRNA) that are processed in a two-step process by a DICER-Like (DCL) ribonuclease, typically DCL1, to give rise to a miRNA-5p/miRNA-3p duplex (Kurihara and Watanabe 2004). Even though DCL1 is predominantly involved in the production of miRNAs, there are also examples of endogenous short interfering RNAs (siRNAs) that are generated by DCL1 activity in Arabidopsis, such as certain natural antisense transcript-derived siRNAs (nat-siRNAs) and long siRNAs (lsiRNAs) (Borsani et al. 2005; Katiyar-Agarwal et al. 2006). The miRNA duplex is methylated and exported to the cytoplasm where one miRNA strand is selectively incorporated into an AGO1-containing RNA-induced silencing complex (RISC). This complex interacts with mRNA targets to direct cleavage or to suppress translation.

Plant miRNAs are important regulators of gene expression in diverse developmental processes (Palatnik et al. 2003; Mallory et al. 2004; Jones-Rhoades et al. 2006; Chuck et al. 2007). They are also known to be involved in hormone signal transduction and adaptation to abiotic and abiotic stress (Fujii et al. 2005; Navarro et al. 2006; Jagadeeswaran et al. 2009; Li et al. 2010; Jeong and Green 2013; Seo et al. 2013; Fei et al. 2016; Baldrich and San Segundo 2016; Huang et al. 2016). Most of our knowledge on miRNAs involved in plant immune responses to pathogen infection comes from studies in the interaction of Arabidopsis plants with the bacterial pathogen *Pseudomonas syringae.*

Plants have evolved multiple defense mechanisms to defend themselves from pathogen infection forming the innate immune system. Defense reactions are activated by the recognition of conserved Pathogen Associated Molecular Patterns (PAMPs) by host membrane Pattern-Recognition Receptors (PRR). This recognition triggers the so called PAMP-triggered immunity (PTI) which is effective against most pathogens (Jones and Dangl 2006; Bigeard et al. 2015; Couto and Zipfel 2016). Among others, PTI components include production of reactive oxygen species (ROS), reinforcement of cell wall, activation of protein phosphorylation/dephosphorylation processes, and accumulation of antimicrobial proteins. The induction of a group of genes known collectively as *Pathogenesis-Related* (*PR*) genes is a ubiquitous response of plants to pathogen infection (Van Loon et al. 2006). Damage associated molecular patterns (DAMPs), released from the plant cell wall following damage caused by the pathogen, also induce plant defense responses. However, certain pathogens are able to suppress these basal resistance mechanisms by delivering effector proteins that can suppress PTI responses into the host cell. As a countermeasure, these microbial effectors are recognized by plant disease resistance proteins (R proteins), establishing the so-called effector-triggered immunity (ETI). Plants also produce a variety of secondary metabolites as natural protection against microbial pathogens. Among them are phytoalexins which are low molecular weight compounds with antimicrobial activity and structural diversity (e.g. flavonoids, terpenoids, indole phytoalexins) (Ahuja et al. 2012; Schmelz et al. 2014).

Rice is one of the most important crops worldwide and a primary source of food for more than a half of human population. Rice blast caused by the fungus *Magnaporthe oryzae* is one of the most devastating fungal disease of cultivated rice worldwide (Wilson and Talbot 2009). Rice is also the model plant for research in monocotyledonous species with a sequenced genome (Yu et al. 2002; Goff et al. 2002). Evidence in the literature supports dramatic variations in the rice miRNA population during *M. oryzae* infection or treatment with *M. oryzae* elicitors (Campo et al. 2013; Baldrich et al. 2015; Li et al. 2016). Although an important fraction of the rice miRNA transcriptome has been reported to be responsive to *M. oryzae* infection or treatment with *M. oryzae* elicitors (Campo et al. 2013; Li et al. 2014; Baldrich et al. 2015), a role for these pathogen-regulated miRNAs has been demonstrated for only a few of them. In particular, miR7695, miR160, miR398 were reported to be positive regulators of the rice response to *M. oryzae* infection, whereas miR169 negatively regulates immunity against this fungus (Campo et al. 2013; Li et al. 2014; Li et al. 2017).

Regarding DCL1, a major miRNA processing component, 3 loci encoding DCL1 proteins are identified in the rice genome, *OsDCL1a*, *OsDCL1b* and *OsDCL1c*. Previous studies revealed that loss-of-function of *OsDCL1a* by RNA interference (*dcl1a-IR* lines) results in abnormal shoot and root development with eventual growth arrest for the strongest RNAi lines (Liu et al. 2005). Later on, it was reported that silencing of *OsDCL1* enhances resistance to the rice blast fungus (Zhang et al. 2015). Contrary to this, a phenotype of susceptibility to pathogen infection is observed in Arabidopsis *dcl1* mutants, these plants showing enhanced susceptibility to infection by bacterial (*P. syringae*) and fungal (*Botrytis cinerea*) pathogens (Navarro et al. 2008; Seo et al. 2013; Weiberg et al. 2014). The *DCL1*-mediated mechanisms underlying these phenotypes of disease resistance or susceptibility in either rice or Arabidopsis remain, however, unknown.

The goal of this research was to investigate the role of *OsDCL1* in rice immunity against fungal pathogens. Towards this end, *OsDCL1a* activation mutants were identified and characterized (named as *dcl1a-Ac* mutants). We show that *OsDCL1a* activation enhances susceptibility to infection by *M. oryzae* (hemibiotroph) and *Fusarium fujikuroi* (necrotroph), the causal agents of the rice blast and bakanae disease, respectively. Susceptibility to pathogen infection in *dcl1a-Ac* plants is associated to a weaker induction of defense gene expression. No negative impact was observed in plant growth performance of *Osdcl1a-Ac* plants. The mRNA transcriptome and miRNAome of *dcl1a-Ac* plants were obtained and compared to those of wild-type plants. This study revealed that *OsDCL1a* activation has an important impact on the expression of genes involved in two processes: ROS detoxification and synthesis of diterpene phytoalexins. In the absence of pathogen infection, *dcl1a-Ac* plants accumulate high levels of the superoxide anion $O_2^{\cdot -}$, these plants being also more sensitive to methyl viologen-induced oxidative stress. Down-regulation of genes involved in the biosynthesis of terpenoid phytoalexins is a feature of *dcl1a-Ac* plants. Together, results here presented support that *OsDCL1a* plays an important role in rice immunity and expands our knowledge about the molecular mechanism and processes involved in blast resistance.

Material and Methods

Plant and fungal material

Plants (*Oryza sativa*) were grown at 28ºC/22ºC day/night with a 16h light/8h dark cycle. Rice genotypes used in this study were: *O. sativa japonica* cv Tainung 67 (TN67), *DCL1a* mutants (M0066754, M0040827) from the Taiwan Rice Insertional Mutant collection (TRIM; http://www.trim.sinica.edu.tw), and *dcl1a-IR* lines (Liu et al. 2005). Genotyping of *dcl1a-Ac* mutants was carried out by PCR on genomic DNA using *DCL1a*-specific primers in combination with a T-DNA-specific primer located at the left border of the T-DNA (**Supplemental Table 1**).

The fungus *M. oryzae* (strain *Guy-11*) was grown as previously described (Campos-Soriano et al., 2012). The fungus *Fusarium fujikuroi* (isolate 297) was grown for 15 for days on PDA medium. Fungal spores were collected by adding sterile water to the surface of the mycelium and adjusted to the appropriate concentration.

Infection assays and elicitor treatment

Infections with *M. oryzae* were carried out by spraying leaves of three weeks-old rice plants with a spore suspension (5 x 10^5 spores/mL). In all experiments, mock inoculations were performed. Development of disease symptoms was followed with time. Lesion area was determined using digital imaging software (Assess 2.0, American Phytopathological Society). For infection experiments with *F. fujikuroi***,** seeds were sterilized with sodium hypochlorite (30%, for 30 min), and placed on Murashige and Skoog (MS) media without sucrose. Seeds were pregerminated for 24 h and then inoculated with a suspension of *F. fujikuroi* spores (1 x 10^6 spores/mL; 10 µL per seed). Three independent infection experiments with each one fungus were performed (at least 24 plants per genotype in each experiment). Statistically significant differences among genotypes were determined by one-way ANOVA test. Quantification of fungal DNA on infected leaves was carried out by qPCR using specific primers for the 28S DNA gene of the corresponding fungus (Qi and Yang 2002; Jeon et al. 2013). Primers are listed in **Supplemental Table 1**. A standard curve using fungal DNA was prepared for quantification of fungal DNA in the infected leaf samples. For elicitor treatment, three weeks-old wild-type plants where sprayed with an elicitor suspension obtained by autoclaving and sonicating *M. oryzae* mycelium (300 µg/mL) (Casacuberta et al. 1992).

RT-qPCR and stem-loop RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from DNAse-treated total RNA (1 µg) with SuperScript III reverse transcriptase (Invitrogen GmbH) and oligo-dT. RT-qPCRs were performed in optical 96-well plates in a Light Cycler 480 (Roche) using SYBR Green. The *cyclophilin 2* gene (Os02g02890) was used to normalize the transcript level in each sample.

Treatment with methyl viologen, pigment quantification and determination of the superoxide ion

Approximately 2 cm leaf segments were treated with methyl viologen solution (10 µM). Leaf samples were maintained at room temperature in the dark for 12 hours. Next, leaves were incubated at 28ºC at a 16h/8h photoperiod cycle for 3 days. Chlorophylls and carotenoids were extracted and quantified spectrophotometrically (Lichtenthaler and Buschmann 2001). For histochemical detection of O_2^-, leaf sections of approximately 2 cm in length were stained with nitroblue tetrazolium (NBT) as previously described (Campo et al. 2014). Three biological replicates with three technical replicates each were performed. Statistical analyses were performed using ANOVA-test.

RNAseq and small RNAseq

Libraries were prepared from leaves of three-week old wild-type (segregated azygous) and *dcl1a-Ac* plants (two biological replicates per genotype). Indexed libraries were prepared from 1 µg/ea of purified RNA (TruSeq Stranded mRNA Sample Prep Kit, Illumina). Libraries were quantified using the Agilent 2100 Bioanalyzer (Agilent Technologies) and pooled such that each index-tagged sample was present in equimolar amounts, with final concentration of the pooled samples of 2nM. The pooled samples were subject to cluster generation and sequencing using an Illumina HiSeq 2500 System (Illumina) at Genomix4life S.R.L. (Baronissi, Salerno, Italy) in a 2x100 paired-end format at a final concentration of 8 pmol. The raw sequence files generated underwent quality control analysis using FastQC v0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimming and removal of adapters was performed by Trimmomatic v0.33 (Bolger et al. 2014) (minimum quality score 35, minimum length 25). The obtained reads were then mapped against the *Oryza sativa* reference genome (MSU 7.0) with STAR (v2.4.0j) (Dobin et al. 2013) providing the reference gene annotation file with known transcripts (RGSP 7.0). Alignment files were filtered to remove reads with MAPQ <30. FeatureCounts v1.4.5-p1 (Liao et al. 2014) was used to perform read summarization at gene level, with the strand-specific option “reversely stranded”. Statistical analysis of the read counts was performed with R, using the HTSFilter package to remove lowly expressed genes (Rau et al. 2013) and the edgeR package to perform differential expression analysis (Robinson et al. 2010). Gene Ontology enrichment analysis of the differentially expressed genes was performed with the AgriGO webtool (http://bioinfo.cau.edu.cn/agriGO/) (Du et al. 2010).

Results

Identification and characterization of activation-tagged *DCL1a* mutants

Knowing that *DCL1* silencing has a negative impact on plant growth, and to investigate the contribution of DCL1 in disease resistance with no influence of intrinsic developmental cues, we searched for *DCL1* activation mutants in publicly available mutant collections. Two T-DNA tagged lines carrying the T-DNA insertion nearby *OsDCL1a*, lines M0066754 and M0040827, were identified in the activation/knockout Taiwan Rice Insertion Mutant (TRIM) collection (Hsing et al. 2007; http://trim.sinica.edu.tw) (**Fig. 1A**). These mutants were generated in the Tainung 67 (TN67, *japonica*) background. The T-DNA insertion site in each TRIM mutant line was confirmed by PCR using combinations of gene- and T-DNA-specific primers followed by DNA sequencing of the PCR products. Homozygous, hemizygous and azygous plants were identified (**Supplemental Fig. 1A, B**).

The T-DNA used for generating the TRIM library contains eight tandem repeats of the cauliflower mosaic virus *35S* promoter (*CaMV35*) adjacent to the right border, which can activate the expression of genes located around the T-DNA insertion sites. As it is shown in **Fig. 1A** (right panel), *OsDCL1a* transcript levels were significantly higher in leaves from each mutant line compared with azygous (segregated progeny) and wild-type TN67 plants, indicating that they are activation mutants for *OsDCL1a* (hereinafter referred to as *dcl1a-Ac#1 and dcl1a-Ac#2*). As expected, *OsDCL1a* expression was lower in *OsDCL1a* RNAi plants (*dcl1a-IR*) plants compared with its parental genotype (*O. sativa* cv Nipponbare; Liu et al. 2005) (**Fig. 1A,** right panel). Quantitative PCR (qPCR) on genomic DNA revealed that each one of the *dcl1a-Ac* mutants has a single copy of the T-DNA inserted in its genome (**Supplemental Table 2**). Importantly, there were no obvious phenotypic differences between *dcl1a-Ac* mutant and wild-type (azygous and TN67) plants grown under controlled greenhouse conditions (**Supplemental Fig. 1C**).

We considered the possibility that the *CaMV35S* enhancer sequences in the T-DNA might activate the expression of genes located nearby the T-DNA insertion site other than *OsDCL1a*. An examination of the T-DNA insertional site flanking genes identified one gene, the *nascent polypeptide-associated complex subunit alpha* gene, upstream of the *OsDCL1a* locus (**Fig. 1A**). However, transcripts for the *nascent polypeptide-associated complex* subunit alpha gene accumulated at equivalent levels in mutant and control plants (azygous, wild-type) (**Supplemental Fig. 1D**).

Susceptibility to infection by fungal pathogens in *dcl1a-Ac* plants

To investigate the functional relevance of *OsDCL1a* activation in disease resistance, *dcl1a-Ac* plants were examined for resistance to infection by the rice blast fungus *M. oryzae.* The *dcl1a-Ac* plants consistently exhibited higher susceptibility to *M. oryzae* infection compared to control plants (**Fig. 1B**). Susceptibility of *dcl1a-Ac* to blast was confirmed by measuring the average lesion area in the infected leaves of these plants, as well as by quantitative PCR measurement of fungal DNA, an indicator of fungal biomass in the infected leaves (**Fig 1B,** right panels). The *dcl1a-Ac#2* mutant plants also exhibited higher susceptibility to *M. oryzae* infection compared to control plants (**Supplemental Fig. 2**). Increased susceptibility to blast infection in *dcl1a-Ac* plants is consistent with results previously reported on resistance to *M. oryzae* in *dcl1a-IR* lines (Zhang et al. 2015). However, whereas *dcl1a-IR* plants have abnormal growth, the *dcl1a-Ac* plants grew and developed normally.

To get further insights into the mechanisms underlying susceptibility to *M. oryzae* infection in *dcl1a-Ac* plants, we examined the expression pattern of the defense genes *OsPR1a* and *OsPBZ1* (a *PR10* family member) in wild-type and *dcl1a-Ac* plants. These genes are widely used as indicators of induction of rice defense responses during pathogen infection, including *M. oryzae* infection (Midoh and Iwata 1996; Agrawal et al. 2001). As expected, *OsPR1a* and *OsPBZ1* expression was induced in wild-type plants during *M. oryzae* infection (**Fig. 1C**). Although *OsPR1a* and *OsPBZ1* expression was also activated by fungal infection in *dcl1a-Ac* plants, their expression was induced at a much lower level in mutant plants than in wild-type plants at all the time points of infection here examined, indicating that *DCL1a* activation compromises *M.oryzae*-related defense responses in rice. A reduced induction of defense gene expression correlates well with the observed phenotype of susceptibility in *dcl1a-Ac* plants.

We also examined disease resistance of *dcl1a-Ac* plants against the necrotrophic fungus *Fusarium fujikuroi*. This fungus is the causal agent of bakanae, an important seed-borne disease of rice (Wulff et al. 2010). As compared to wild-type segregated azygous plants, *dcl1a-Ac* seedlings grew poorly and their roots turned necrotic upon *F. fujikuroi* inoculation (**Figure 1D**). Quantification of fungal biomass revealed higher fungal growth in roots of *dcl1a-Ac* plants compared to wild-type plants (**Fig. 1D,** right panel), thus, confirming that *dcl1a-Ac* plants are more susceptible to infection by *F. fujikuroi*.

Together, results obtained in infection assays demonstrated that *OsDCL1a* activation enhances susceptibility to infection by hemibiotrophic (*M. oryzae*) and necrotrophic (*F. fujikuroi*) fungal pathogens in rice, and that disease susceptibility in *dcl1a-Ac* plants is associated to a weaker induction of defense gene expression during pathogen infection.

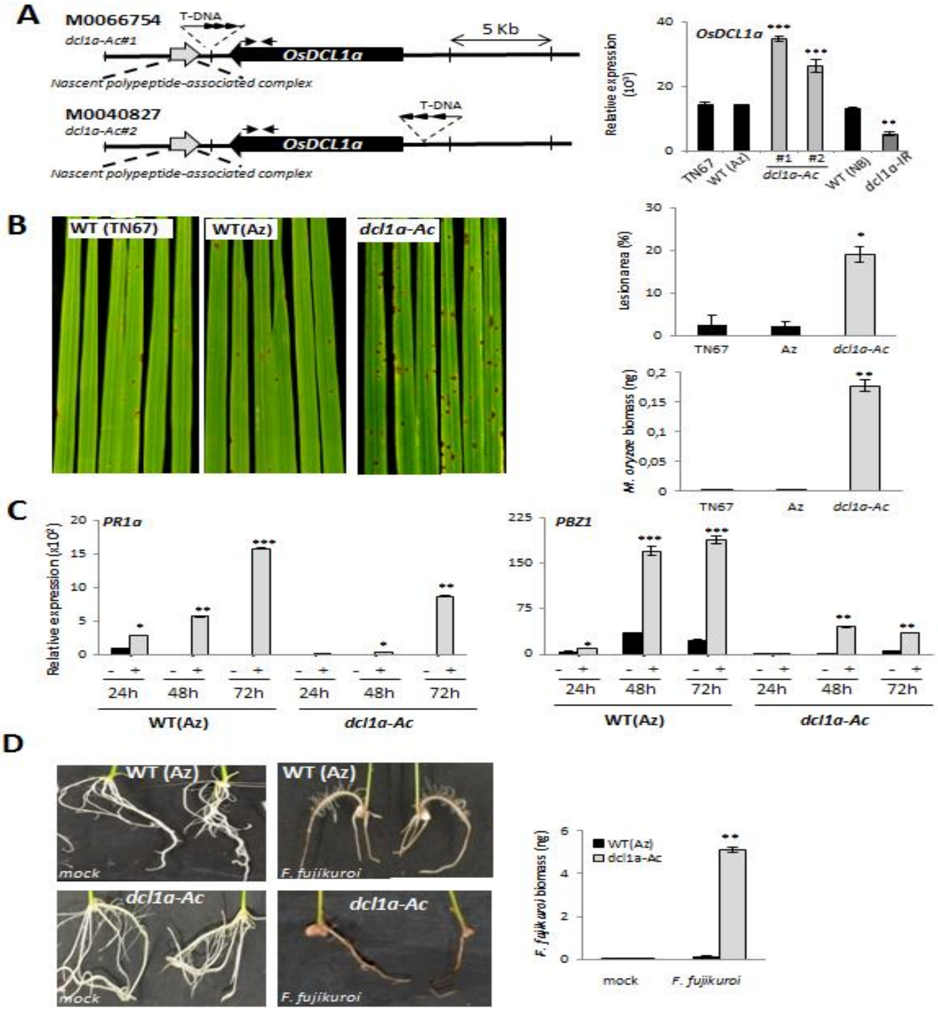


Figure 1. **Characterization of the *OsDCL1a* mutant. A.** Representation of the rice mutants (M0066754, M0040827). T-DNA insertion is shown and black triangles correspond to the CaMV35S enhancer. Black arrow represents the *DCL1a* locus pointing in the direction of transcription. The nearby gene (*nascent polypeptide-associated complex subunit alpha*) is indicated with a grey arrow. Quantitative PCR (RT-qPCR) gene-specific primers for *OsDCL1a* are indicated with black arrows on *OsDCL1a* sequence**.** Accumulation of *DCL1a* was determined by RT-qPCR at 3-leaves plant stage. The *Cyclophylin2* (Os02g02890) was used for normalization. **B.** Phenotype of wild-type, azygous and *dcl1a-Ac* plants 7 days post *M. oryzae* infection. Lesion area and *M. oryzae* biomass were measured **C.** Defense gene expression (*PR1a* and *PBZ1*) measured by RT-qPCR at different time points in *M. oryzae*-infected plants and mock-inoculated. **D.** Phenotype of azygous and *dcl1a-Ac* roots 7 days post *F. fujikuroi* inoculation. Fungal biomass was measured. Histograms show the mean ± SD (***, p-value ≤ 0.001;**, p-value ≤ 0.01; *, p-value ≤ 0.05, determined by ANOVA).

Expression of *DCL* genes in *dcl1a-Ac* plants

In plants, the *DCL* gene family typically comprises four members, *DCL1* to *DCL4*, which have distinct functions in miRNA and siRNA biogenesis (Song et al. 2012; Meng et al. 2014). A fifth *DCL*, *DCL5* (previously named *DCL3b*), appears to have evolved in monocots (Margis et al. 2006; Fei et al. 2013). As previously mentioned, the rice genome contains three *DCL1* genes (*OsDCL1a*, *OsDCL1b*, *OsDCL1c*). *OsDCL1a* is most closely related to *AtDCL1a* from a structural and functional point of view, and *OsDCL1a* silencing impairs miRNA biogenesis in rice (Liu et al. 2005). Additionally, the rice genome has two *DCL2* paralogs with almost identical sequences (*DCL2a/b*), and unique *DCL3* (*OsDCL3a*), *DCL4* and *DCL5* genes (Kapoor et al. 2008; Margis et al. 2006; Fei et al. 2013). Regarding the expression of rice *DCL* genes, *OsDCL1a*, *OsDCL2a/b* and *OsDCL3a* are known to be ubiquitously expressed in vegetative tissues during plant development, but their expression is markedly reduced during reproductive development. As for the low expressing *DCL* genes (*OsDCL1b, OsDCL1c,* and *OsDCL5*), they exhibit inflorescence, panicle- and/or early seed-specific expression (Kapoor et al. 2008). The *OsDCL1a* gene was found to be the most highly expressed *OsDCL1* gene in leaves of three-week old wild-type rice plants (**Supplemental Fig. 3**). As expected, *OsDCL1a* expression further increased in *dcl1a-Ac* plants, but no significant alterations in the expression of any of the other *DCL* genes could be observed in *dcl1a-Ac* plants compared to wild-type plants (**Supplemental Fig. 3**).

The apparently negative effect of *OsDCL1a* activation on resistance to fungal infection prompted us to investigate whether *OsDCL1a* expression itself is regulated as part of host response to pathogen infection. This analysis revealed induction of *OsDCL1a* expression in wild-type plants in response to *M. oryzae* infection (at 24h, 48h and 72 hpi) (**Fig. 2A, left panel**). A similar trend in *OsDCL1a* expression, that is up-regulation, was observed after treatment with elicitors obtained by autoclaving and sonicating *M. oryzae* mycelium (**Fig. 2A, right panel**). Regarding other rice *DCL* genes, a different response to *M. oryzae* infection was observed depending on the family member. Whereas *OsDCL1a* and *OsDCL1b*, *OsDCL2a/b* and *OsDCL3a* expression was induced, *OsDCL4* expression was repressed by *M. oryzae* infection, and *OsDCL5* was not affected (at least at the time point here examined, 72hpi) (**Fig. 2B**).

From these results it is concluded that pathogen infection is accompanied by alterations in the expression of rice *DCL* genes, namely *OsDCL1a, OsDCL1*b, *OsDCL2* and *OsDCL3a*. Knowing that these genes are involved in small RNA biogenesis pathways, this observation anticipates important small RNA-mediated transcriptional reprogramming of gene expression as part of the rice response to infection by the fungal pathogen *M. oryzae*.

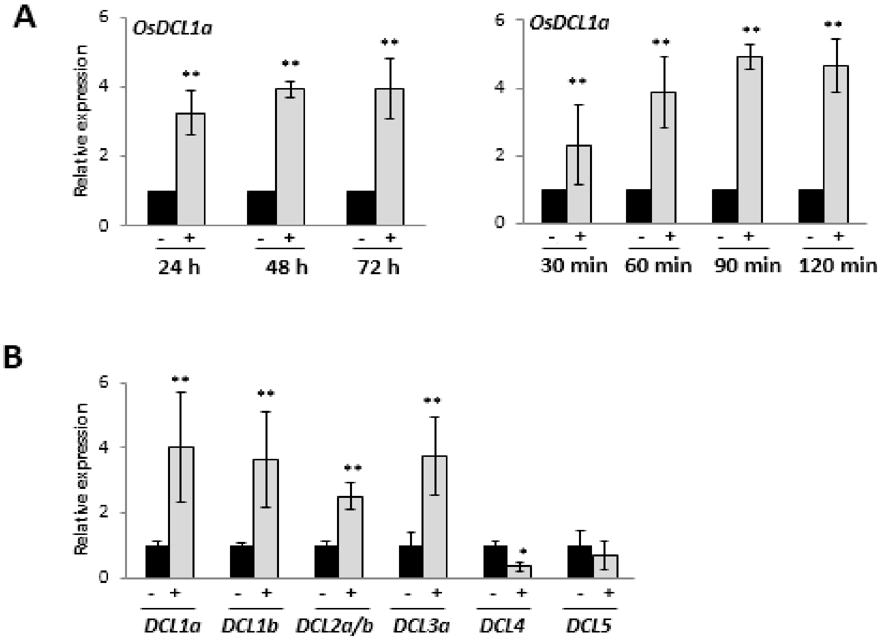


Figure 2. Expression of *DCL* genes during *M. oryzae* recognition. A. Relative expression measured by RT-qPCR of *OsDCL1a* at control and rice blast infection (5x10^5 spores/mL) and control and *M. oryzae* elicitor treatment in wild-type (azygous) plants. **B.** Relative expression of *DCL* genes at control and 72 h after infection with *M. oryzae* spores. Histograms show the mean ± SD. Differences between lines in each time point are marked according to ANOVA (**, p-value ≤0.01; *,p-value≤0.05).

Transcript profiling of *dcl1a-Ac* mutant plants

To investigate *OsDCL1a*-mediated alterations in the rice transcriptome, we conducted RNASeq analysis of *dcl1a-Ac* and wild-type (segregated azygous) plants. RNA was obtained from leaves of 3 weeks-old plants. Illumina Solexa sequencing produced 39’6 and 31 million reads in wild-type and *dcl1a-Ac* plants, respectively (**Supplemental Table 3**). The processed RNA-Seq reads were mapped to the rice genome (*O. sativa* cv Nipponbare MSU 7.0). For the calling of differentially expressed (DE) genes, a fold change value of 2.0 was used as cut-off score, with the False Discovery Rate (FDR) threshold set to 0.05. A total of 216 genes were found to be differentially expressed (DE) in *dcl1a-Ac* plants relative to wild-type plants, most of them being down-regulated in *dcl1a-Ac* plants) (155 genes down-regulated; 61 genes up-regulated) (**Fig. 3A; Supplemental Tables 4 and 5**). A gene ontology (GO) functional analysis revealed that, among those down-regulated genes in *dcl1a-Ac* plants, an important number of genes grouped into the categories of “Signalling”, “Metabolism”, and “Biotic Stress” (28%, 19% and 14 %, respectively) (**Fig. 3B,** left panel).

A remarkable difference was observed in the distribution of DE genes in functional categories between up-regulated genes and down-regulated genes in *dcl1a-Ac* plants (**Fig. 3B,** right panel). For instance, genes associated with “Biotic Stress” were not represented in the set of up-regulated genes in *dcl1a-Ac* plants, whereas genes involved in oxidative stress were highly represented. On the other hand, genes involved in “Signalling” were less represented in the group of up-regulated genes than in down-regulated genes (**Fig. 3; Supplemental Tables 4 and 5**). DE genes in *dcl1a-Ac* plants were also classified according to their molecular functions using the AgriGO analysis tool (Du et al. 2010). Important differences were observed in the categories of protein kinase activity and oxidoreductase (monooxygenase) activities (**Supplemental Fig. 4**). For instance, we noticed that the expression of an important number of receptor-like kinases was down-regulated in *dcl1a-Ac* plants compared to wild-type plants. The subfamily of cell *Wall-Associated Kinases* (*WAKs*) was the most highly represented group of down-regulated receptor kinase genes (up to 17 *WAKs* were down-regulated in *dcl1a-Ac* plants) (**Supplemental Table 4**). WAKs are involved in perception of PAMPs and DAMPs for the activation of defense-associated responses, and overexpression of *WAK* genes increases resistance to pathogen infection, including *M. oryzae* in rice (Li et al. 2009; Delteil et al. 2016). Two *brassinosteroid insensitive 1 receptor kinase* (*BRI1*) genes were also down-regulated in *dcl1a-Ac* plants (**Supplemental Table 4**), these genes being also involved in recognition of PAMPs and activation of plant immune responses. Genes typically associated to disease resistance and defense mechanisms were also down-regulated in *dcl1a-Ac* plants, such as several *R* genes and the *OsWRKY47* transcription factor. Previous studies have shown that overexpression of *OsWRKY47* in rice is accompanied by up-regulation of *PR10* and blast resistance (Wei et al. 2013). In agreement with this, *dcl1a-Ac* plants showed down-regulation of both *OsWRKY47* and *PR10* expression. Among the genes that are expressed at a lower level in *dcl1a-Ac* compared to wild-type plants there were genes involved in the biosynthesis of antifungal compounds, such as *Agmantine hydroxycinnamoyltransferase1* (for production of antifungal hydroxycinnamoylagmantine derivatives) and *strictosidine synthase* (for production of alkaloids) (**Supplemental Table 4**).

To note, genes encoding enzymes involved in oxidation-reduction reactions were highly represented in the group of misregulated genes in *dcl1a-Ac* plants (up-regulated and down-regulated genes). They included several peroxidases and cytochrome P450 monooxygenases (CYPs) (**Supplemental Tables 4 and 5**). CYPs catalyze the oxidation of many substrates for the production of several metabolites, these enzymes being involved in the production of phytoalexins and phytohormones.

Validation of selected DE genes in *dcl1a-Ac* vs wild-type plants was carried out by RT-qPCR. We selected DE genes classified in the categories of “Signalling”, “Biotic Stress” and “Oxidative Stress. We further extended this analysis by determining the expression of these genes under non-infection and infection conditions (e.g. 72 hpi with M. *oryzae*). In the absence of pathogen infection, Receptor kinase genes (*Receptor kinase 5*, *OsWAK47, OsWAK14*), disease resistance (*RPM1*) and defense genes (*PR10*, *BetV*) were expressed at a significantly lower level in *dcl1a-Ac* plants compared to wild-type plants (**Fig. 3C,D,** black bars). Whereas *Prx83* was down-regulated in *dcl1a-Ac* plants, two other peroxidase genes were up-regulated (*Prx14* and *Prx34*) in these plants in the absence of pathogen infection (**Fig. 3E,** black bars). Together, these results indicated good correlation between RT-qPCR analysis and RNA-Seq data.

Upon pathogen challenge, *RPM1*, *PR10*, and *BetV* reached a lower level of induction in *dcl1a-Ac* compared to wild-type plants, whereas the fungal-responsiveness of *Receptor Kinase 5* was found to be compromised in the *dcl1a-Ac* plants (**Fig. 3C, D**). Regarding *Prx14* and *Prx34*, their expression was more strongly induced in the mutant plants (**Fig. 3E**). The observed misregulation of host genes, including the lower induction of defense-related genes in response to pathogen infection (e.g. *receptor kinase* genes, *R* genes, and *PR* genes), might well contribute to disease susceptibility in *dcl1a-Ac* plants.

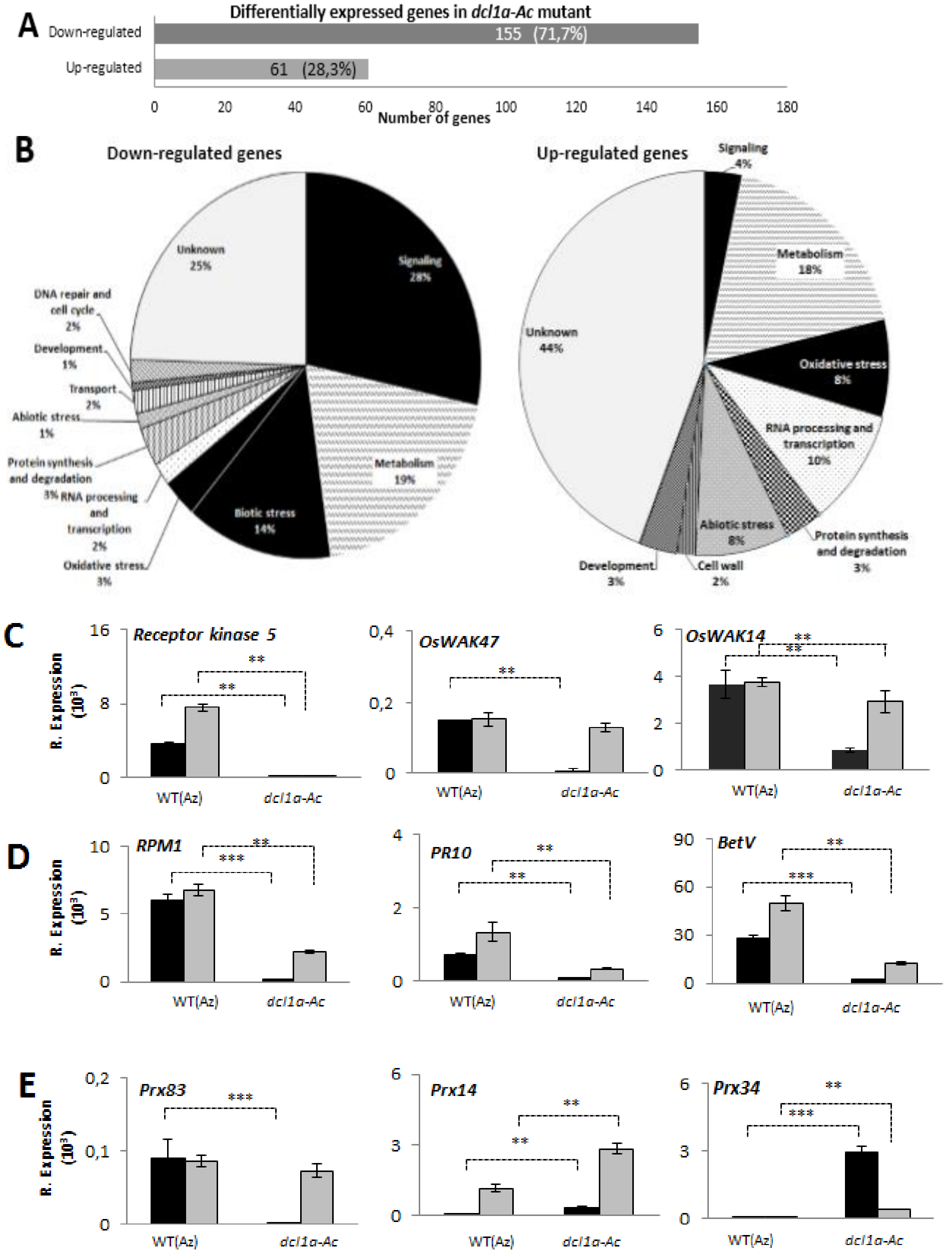


Figure 3. Misregulated genes in *dcl1a-Ac* line. A. Number of down-regulated and up-regulated genes in *dcl1a-Ac* line compared to wild-type azygous plants. **B.** Representation of functional categories of down-regulated and up-regulated genes in *dcl1a-Ac* line using Agrigo. **C-E.** RT-qPCR validation of genes falling into signalling (C), biotic stress (D) and oxidative stress (E) categories. Histogram show the mean ± SD according to ANOVA (***, p-value ≤ 0.001;**, p-value ≤ 0.01).

DCL1 activation leads to reduced expression of phytoalexin biosynthesis genes

Diterpenoid phytoalexins are the major phytoalexins in rice, which are classified into five groups based on carbon skeleton: momilactones (A and B), oryzalexins (A to F), oryzalexin S, phytocassenes (A to E), and *ent*-10-oxodepressin (Ahuja et al. 2012; Inoue et al. 2013; Yamane 2013). Interestingly enough, our RNASeq analysis revealed that genes involved in the biosynthesis of the various diterpenoid phytoalexins are down-regulated in *dcl1a-Ac* plants compared to wild-type plants (**Fig. 4A, B; Supplemental Table 4**). They included: genes involved in the biosynthesis of momilactones A and B (*CPS4*, *CYP99A2*, *CYP99A3*, *MAS1*, *MAS2, CYP76M5*), oryzalexin S (*CPS4*, *KSL8*), oryzalexins A to F (*CPS2*, *KSL10*, *CYP701A8*, *CYP76M5*, *CYP76M6*, *CYP76M8*), and phytocassenes A to E (*CPS2*, *KSL7*, *CYP71Z7*, *CYP76M8*, *CYP701A8*) (**Fig. 4A, B**). Furthermore, the expression of diterpenoid phytoalexin biosynthetic genes was induced to a lower extend in *dcl1a-Ac* plants than in wild-type plants during *M. oryzae* infection (24h, 48, and 72 hpi) (**Fig. 4C**).

In addition to diterpenoid phytoalexins, a flavonoid phytoalexin, the so called sakuranetin, which has antifungal activity on *M. oryzae* growth has been described to accumulate in resistant rice plants after *M. oryzae* infection (Dillon et al. 1997; Hasegawa et al. 2010). Our RNASeq analysis revealed that *isoflavone reductase* encoding one of the key enzymes in isoflavonoid phytoalexin biosynthesis was down-regulated in *dcl1a-Ac* plants (**Supplemental Table 4**). Overexpression of a isoflavone reductase-like gene (*OsIRLs*) in rice has been shown to confer tolerance to ROS and treatment with methyl viologen, a ROS-generating agent (Gon et al. 2010).

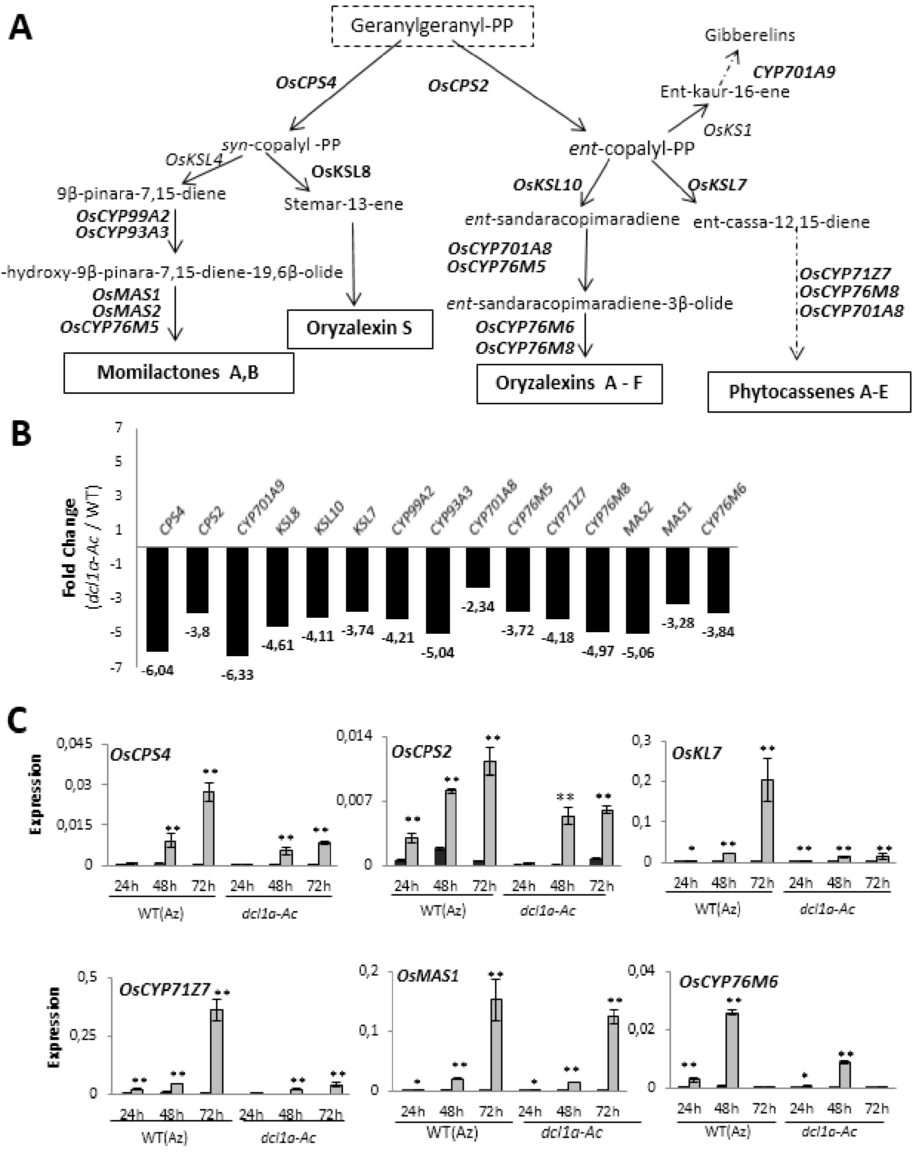


Figure 4. Diterpenoid phytoalexin transcriptome analysis. A. Diterpenoid phytoalexins biosynthesis pathway. Misregulated genes in *dcl1a-Ac* line are marked in bold. Final phytoalexin molecules are framed. B. Fold-change representation of misregulated biosynthesis genes. C. RT-qPCR validation of phytoalexins synthesis gene expression in wild-type (azygous) and *dcl1a-Ac* lines. Histogram show the mean ± SD according to ANOVA (**, p-value ≤ 0.01; *,p-value ≤0.05).

Reduced tolerance to oxidative stress in *dcl1a-Ac* plants

ROS are constantly being generated during normal plant growth and development, and unbalance between ROS generation and safe detoxification generates oxidative stress in plants. As previously mentioned, a substantial number of genes encoding enzymes that function in oxidation-reduction reactions are misregulated in *dcl1a-Ac* plants, including peroxidase, glutathione-S-transferase and *CYP* genes (**Supplemental Tables 4 and 5**). We then hypothesized that *DCL1* activation might affect ROS detoxification systems and/or ROS homeostasis which, in turn, might affect redox-dependent cellular processes. ROS includes superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2), of which the $OH^{\bullet}$ radical is the most reactive molecule. Furthermore, $O_2^{\bullet -}$ and H_2O_2 can react with each other in the presence of metal ions, such as iron, to form the more reactive hydroxyl radicals $OH^{\bullet}$ and OH^- through the Haber-Weiss and Fenton reactions. Hydroxyl radicals are highly reactive and interact with all biological molecules leading to cellular damages.

We investigated whether transcriptome affectation caused by *DCL1* activation has an effect in the host ROS detoxification system. The efficacy of the endogenous ROS scavenging system was examined using the ROS-generating reagent, methyl viologen (MV). This compound acts as an inhibitor of photosynthesis and promotes the formation of superoxide anion ($O_2^{\bullet -}$) which results in reduced chlorophyll content and discoloration in MV-treated leaves (Camejo et al. 2016). As it is shown in **Figure 5A**, leaves of *dcl1a-Ac* plants were greatly affected by treatment with MV, and the chlorophyll content was markedly reduced in leaves of MV-treated *dcl1a-Ac* plants compared to MV-treated wild-type plants. Carotenoids are also able to detoxify ROS, and treatment with MV results in a higher reduction of carotenoid content in leaves of *dcl1a-Ac* plants than in leaves of WT plants (**Fig. 5A,** right panel). Finally, when examining the effect of treatment with MV in knock-down *dcl1a-IR* plants, the reduction in chlorophyll content in these plants was similar to that of its wild-type parental genotype Nipponbare (**Supplemental Fig. 5A**). Although a reduction in carotenoids could be observed in *dcl1a-IR* plants compared to its parental genotype, this difference was not statistically significant.

Finally, we examined $O_2^{\bullet -}$ accumulation in *dcl1a-Ac* and wild-type plants grown under controlled conditions (e.g. in the absence of pathogen infection). For detection of $O_2^{\bullet -}$ in rice leaves we used nitroblue tetrazolium (NBT) staining. To note, *dcl1a-Ac* plants accumulated important levels of $O_2^{\bullet-}$ in their leaves (**Fig. 5B**). As control, leaves of wild-type plants (TN67) were treated with the ROS generating agent H_2O_2 and examined for $O_2^{\bullet-}$ accumulation. Contrary to what is observed in *dcl1a-Ac* plants, the *dcl1a-IR* plants have no visible alterations in $O_2^{\bullet-}$ accumulation (**Supplemental Fig. 5B**).

Altogether, these results indicated that *DCL1a* activation renders the plant more sensitive to oxidative stress caused by treatment with MV and induces $O_2^{\bullet-}$ accumulation in leaves. Disturbances in ROS production and/or scavenging mechanisms might interfere with the normal function of host antioxidant systems which might explain, at least in part, the phenotype of disease susceptibility in *dcl1a-Ac* plants. Further studies are needed to clarify the exact biochemical mechanisms by which *DCL1a* activation stimulates $O_2^{\bullet-}$ accumulation, and possibly, alters ROS homeostasis in rice.

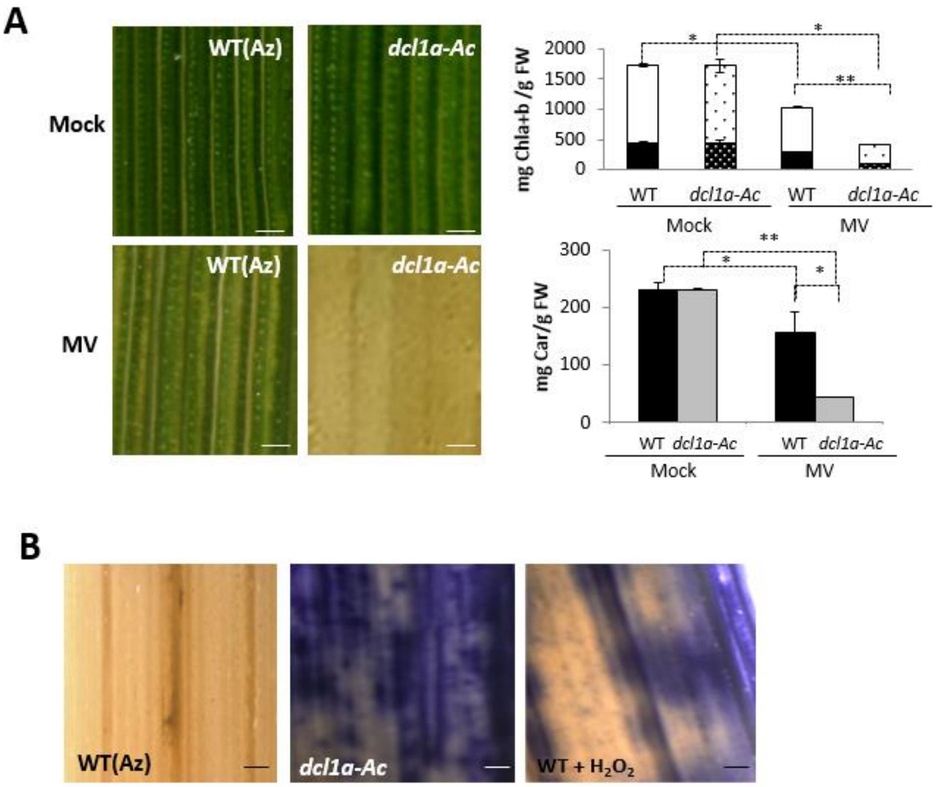


Figure 5. Effect of treatment with methyl viologen (MV) and detection of superoxide ion ($O_2^{\bullet -}$) in *dcl1a-Ac* plants. Histograms show the mean ± SD. Asterisks indicate significant differences; **, $P \leq 0.01$; *, $P \leq 0.05$. **A.** Leaves of three-week old *dcl1a-Ac* (*Osdcl1a-Ac#1*) and wild-type (azygous) plants were treated with MV (10µM). Chlorophyl and carotenoid content was determined after 72 h of treatment. Right panels: quantification of chorophyls (Chla + Chlb) and carotenoids in mock-inoculated and MV-treated wild-type and *dcl1a-Ac* plants. Data shown correspond to wild-type (black, Chla; white, Chlb;) and *dcl1a-Ac* (dotted) plants. **B.** Detection of $O_2^{\bullet -}$ radicals by nitroblue tetrazolium (NBT) staining. As a control, leaves were treated with H_2O_2 for 6 h. Bars represent 250 µm.

Characterization of the miRNAome in the *dcl1a-Ac* mutant

Knowing that the activity of DCL1 is required for processing of miRNA precursors and production of mature miRNAs, we reasoned that *DCL1a* activation might have an impact on the rice miRNAome. Accordingly, small RNA sequencing was performed for the characterization of the miRNA population in leaves of wild-type and *dcl1a-Ac* plants. Two small RNA libraries, representing independent biological replicates of each genotype were prepared (same biological samples than for mRNA transcript profiling). Illumina sequencing of small RNA libraries generated 36 million reads (15 and

21 million reads from wild-type and mutant plants, respectively) (**Supplemental Table 6**). After removal of the adapter sequences and sequences shorter than 15 bp, a total of 32 million reads were obtained (14 and 18 million from wild-type and *dcl1a-Ac* plants, respectively). All unique sequences were aligned to the rice genome (Nipponbare reference genome MSU 7.0), and reads mapping to known non-coding RNA families (rRNAs, tRNAs, small nuclear RNAs and small nucleolar RNAs) were removed. The abundance of small RNAs was calculated as RPKMs (Reads Per Kilobase Million).

Consistent with the distribution of small RNA sizes that is typically observed in plants, the 24 nt small RNA class was the most abundant size class in both genotypes, with the 21 nt small RNAs forming a secondary peak (**Fig. 6A**). In *dcl1a-Ac* plants, however, the small RNA size distribution showed a substantial increase in the 21 nt small RNA class, which was evident when considering both relative abundance and distinct reads (**Fig. 6A**). The observed increase in the 21 nt small RNA population might be due to the fact that DCL1 is involved in the production of almost all canonical 21 nt miRNAs.

A blast search against the miRNA database (miRBase release 21) allowed us to identify known miRNAs present in our small RNA sequencing data. Then, differentially expressed miRNAs were defined as those with changes ≥ 1.5-fold (up-regulated) or ≤ 0.5-fold (down-regulated), and P-value ≤ 0.05. By using these criteria, up to 90 miRNAs corresponding to 61 miRNA families were found to be differentially expressed in *dcl1a-Ac* plants (**Supplemental Table 7**). Although the most obvious trend that could be expected from transcriptional activation of *OsDCL1a* was an enrichment of miRNAs (which are likely to involve DCL1 in their biogenesis), we noticed that miRNAs differentially expressed in *dcl1a-Ac* plants included both up-regulated and down-regulated miRNAs. Representative examples of DE miRNAs in *dcl1a-Ac* plants are shown in **Figure 6B**. The expression of selected miRNAs was validated by stem-loop RT-PCR (ST-RT-PCR), including miRNAs that are up-regulated (miR1431, miR1847, miR2865, miR3982-3p) or down-regulated (miR393, miR396abc, miR398, miR529b) in *dcl1a-Ac* plants (**Fig. 6C, D**). A concordance between the sequencing based profiling and stem-loop RT-PCR was observed, supporting up-regulation and down-regulation of miRNAs in *dcl1a-Ac* plants.

According to small RNASeq data and SL-RT-qPCR analysis miR398 accumulation decreased in *dcl1a-Ac* plants compared to wild-type plants (**Fig. 6D**), which correlated with a reduced level of miR398 precursor transcripts, and increased accumulation of miR398-targeted *Superoxide Dismutase 2* (*SOD2*) transcripts in *dcl1a-Ac* plants (**Supplemental Fig. 6**). Previous studies reported that transgenic rice lines overexpressing *MIR398* exhibit enhanced resistance to *M. oryzae* infection (Li et al. 2014), which is consistent with the observed phenotype of susceptibility to *M. oryzae* infection in *dcl1a-Ac* plants (in which miR398b accumulation is reduced compared to wild-type plants).

Collectively, results here presented demonstrate that *DCL1a* activation results in important perturbations in the rice miRNAome. Presumably, perturbations on miRNA expression patterns might lead to alterations in the expression of the corresponding target genes, which might contribute to susceptibility to *M. oryzae* infection in *dcl1a-Ac* plants.

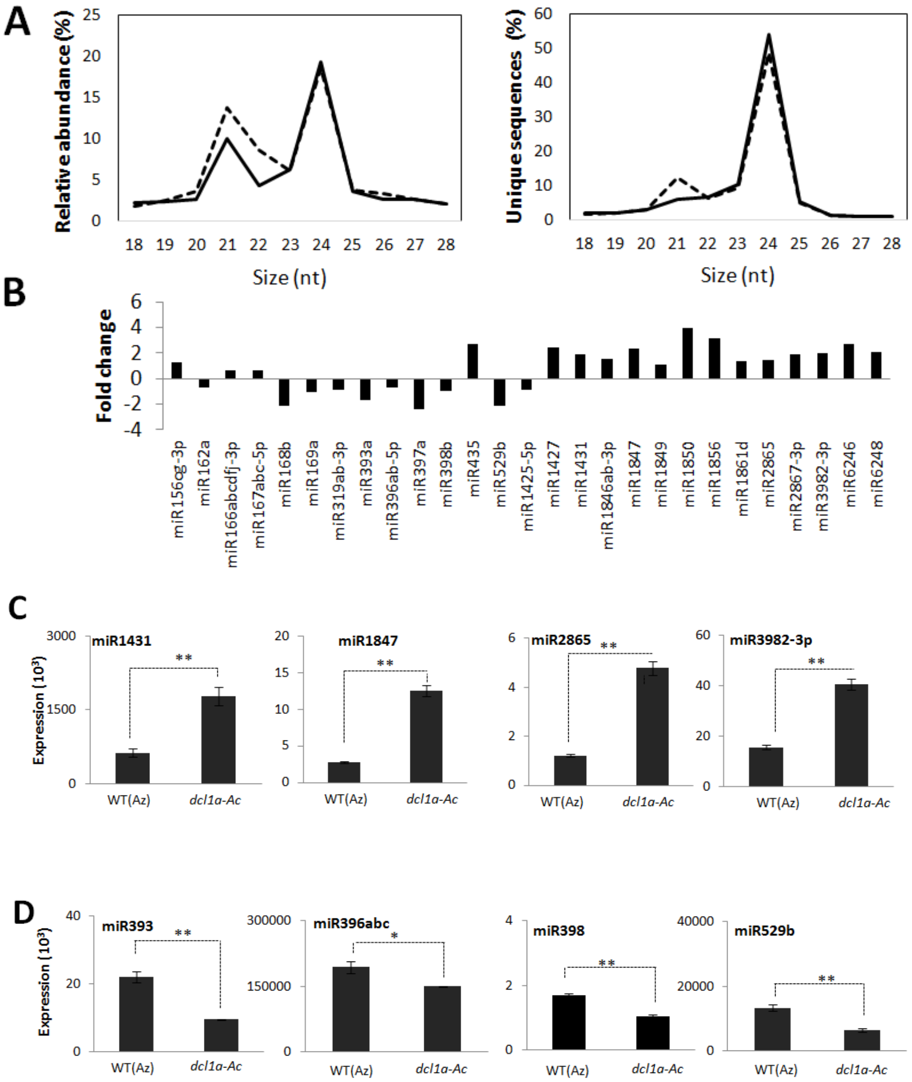


Figure 6. Impact of *DCL1a* activation on the rice leaf miRNAome. Asterisks in C and D indicate significant differences (ANOVA test; **, P ≤ 0.01; *, P ≤ 0.05). **A.** Abundance and unique small RNA sequences for each size class in leaves of wild-type and *dcl1a-Ac* plants (solid and dashed lines, respectively). **B.** Expression profiling of known miRNAs in *dcl1a-Ac* plants relative to wild-type plants. Representative examples are shown. Reads retrieved from the Illumina sequencing datasets for each family member were normalized to the total count of reads obtained in the corresponding library. Fold change was calculated on the basis of normalized reads (RPKM) (*dcl1a-Ac* vs wild-type). **C.** Stem-loop RT-PCR of miRNAs identified as up-regulated in *dcl1a-Ac* plants. **D.** Stem-loop RT-PCR of miRNAs identified as down-regulated in *dcl1a-Ac* plants.

Discussion

In this work, we provide evidence that DCL1a, a component of the miRNA biogenesis pathway, plays a crucial role in rice immunity. Several lines of evidence support this conclusion. First, mutant plants in which *OsDCL1a* expression is activated by T-DNA tagging are more susceptible to infection by hemibiotrophic (*M. oryzae*), and necrotrophic (*F. fujikuroi*) fungal pathogens. Second, susceptibility to pathogen infection is accompanied by a weaker induction of defense-related genes during pathogen infection (e.g. *OsPR1a* and *OsPBZ1*, marker genes of the rice defense response to *M. oryzae* infection). Third, genes involved in the production of diterpenoid phytoalexins for which an antifungal activity against *M. oryzae* has been demonstrated are down regulated in *dcl1a-Ac* mutant plants. Finally, genes involved in redox homeostasis are misregulated in the mutant plants. Additionally, the observation that Os*DCL1a* expression itself is regulated, not only by *M. oryzae* infection, but also by treatment with *M. oryzae* elicitors in wild-type plants supports that *OsDCL1* is a component of PTI responses in rice. Collectively, these results indicated that *OsDCL1a* functions as a negative regulator of the rice defense response. These findings are in agreement with Zhang et al. (2015), who reported enhanced resistance to *M. oryzae* infection by RNA-interference-mediated silencing of *OsDCL1a* (*dcl1a-IR* plants). However, whereas rice plants overactivating *DCL1a* developed and grew normally, *dcl1a* silencing causes severe developmental defects in rice (Liu et al. 2005). Transcriptional activation and silencing of *DCL1a* appears not to have opposite effects on host developmental programs.

Whereas *DCL1a* functions as a negative regulator in rice immunity, *DCL1* was reported to act as a positive regulator of immune responses in Arabidopsis. Thus, Arabidopsis *dcl1* mutants show hyper-susceptibility to infection by the bacterial pathogen *P. syringae* (*dcl1-7* and *dcl1-9* mutants) and to the fungal pathogen *B. cinerea* (*dcl1-7* mutant) (Navarro et al. 2008; Li et al. 2010; Weiberg et al. 2014). It is then likely that the regulatory activity of *DCL1a* in rice is different from its Arabidopsis counterpart in determining the outcome of the plant/pathogen interaction. Alternatively, DCL1a might execute its regulatory role through different pathways depending on the type (fungal or bacterial pathogens), or lifestyle of the pathogen (biotrophs, hemibiotrophs, necrotrophs). Further investigation is needed to understand why alterations in

DCL1a expression have a different impact in susceptibility/resistance to pathogen infection in rice and Arabidopsis.

The comparison of the *dcl1a-Ac* and wild-type transcriptomes allowed us to identify *OsDCL1a*-mediated processes with relevance to blast resistance. Under normal growth conditions, *R* genes (*RPM1* and *Verticillium wilt disease resistance* genes), and receptor kinase genes, including many *WAK* receptor kinases, were found to be down-regulated in *dcl1a-Ac* plants. Down-regulation of defense-related receptor kinases suggests that pathogen perception might be extensively affected in these plants which might result in either no detection of the pathogen, suppression of PAMP/DAMP-elicited defense responses, or production of ineffective defense responses in *dcl1a-Ac* plants. Although the relevance of such alterations in susceptibility to *M. oryzae* in *dcl1a-Ac* plants has not been demonstrated in this study, their involvement in resistance to pathogen infection is well documented in other plant species (Boyes et al. 1998; Grant et al. 2000; Fradin et al. 2009). In particular, WAK receptor kinases are known to regulate resistance to *M. oryzae* in rice (Delteil et al. 2016).

Failure to alleviate methyl viologen-mediated oxidative stress supports that protective antioxidant systems do not function properly in *dcl1a-Ac* plants under normal growth conditions. Along with this, *dcl1a-Ac* plants accumulate important levels of the superoxide ion $O_2^{\bullet-}$ in their leaves. Although $O_2^{\bullet-}$ is moderately reactive and does not cause extensive damage by itself, this radical undergoes transformation into the more reactive and toxic $OH^{\bullet}$ which is highly reactive and causes cellular damage. In the absence of infection, however, $O_2^{\bullet-}$ accumulation does not appear to cause negative effects in plant growth. On the other hand, it is well known that ROS production is one of the earliest responses of plant tissues to pathogen attack, the so called oxidative burst. Unless ROS are effectively detoxified, ROS overproduction during pathogen infection would facilitate oxidative damage in the host plant. How DCL1a activation compromises ROS detoxification mechanisms deserves further investigation.

Even more interesting is the fact that genes involved in diterpenoid phytoalexin biosynthesis were the most predominant group of down-regulated genes in *dcl1a-Ac* plants. The accumulation of momilactones and oryzalexins has been shown to be critical to counteract *M. oryzae* infection in rice (Dillon et al. 1997; Umemura et al. 2003). Also, diterpenoid phytoalexin genes show faster and/or stronger induction in resistant rice cultivars than in susceptible cultivars (Hasegawa et al. 2010; Bagnaresi et al. 2012). The observed phenotype of disease susceptibility in *dcl1a-Ac* plants can then be attributed, at least in part, to down-regulation of phytoalexin biosynthesis genes. These findings, not only reinforce the notion that *OsDCL1a* is a negative regulator of immune responses in rice, but also supports a DCL1-mediated regulation of secondary metabolic defense pathways with relevance to pathogen resistance, most probably through regulation of miRNA accumulation.

Characterization of the miRNAome in leaves of *dcl1a-Ac* plants allowed us to identify alterations in the accumulation of specific miRNA families caused by *DCL1a* activation. The observed increase in the accumulation of miRNAs in *dcl1a-Ac* plants is consistent with targeted activation of *OsDCL1* in this mutant. Interestingly, a down-regulation of different *MIR* genes was also observed in *dcl1a-Ac* plants, pointing to factors other than processing of miRNA precursors by DCL1a in controlling the accumulation of rice miRNAs. There are several reasons that can explain no alteration in the accumulation of distinct miRNAs or even the otherwise paradoxical decrease in the amount of miRNAs in *dcl1a-Ac* mutant plants. Thus, miRNA accumulation might be affected by the spatio-temporal expression pattern of genes involved in miRNA biogenesis other than *OsDCL1a*. In addition to processing of precursors by DCL1, the abundance of mature miRNAs might be affected by miRNA stability (which is also dependent on miRNA modifications such as 3' end methylation or nucleotide addition), binding of miRNAs to AGO (which protects miRNAs from degradation), or sequestration by target mimic RNAs. As an additional complexity, evidence exists on auto-regulatory feedback loops between miRNA and target genes, in which target genes can control the level of a miRNA in addition to being regulated by it. The best known example is the transcriptional/translational interlocked feedback loop governing miR168/AGO1 pair function, in which a miR168-guided cleavage of AGO1 and a post-transcriptional stabilization of miR168 by AGO1 occurs (Vaucheret et al. 2006). Here, it is worth mentioning that *OsDCL1* is itself regulated by miR162 (Xie et al. 2003) and that miR162 was found to be down-regulated in *dcl1a-Ac* plants ($\log_2$ FC -0.73). A feedback mechanism by which DCL1a accumulation in *dcl1a-Ac* plants might negatively regulate miR162 level can be envisaged. Other miRNAs accumulating at a lower level in *dcl1a-Ac* plants compared to wild-type plants were miR393 and miR398. In Arabidopsis, miR393 overexpression renders plants more resistant to the biotrophic oomycete *Hyaloperonospora arabidopsidis*, and more susceptible to the necrotrophic fungus *Alternaria brassicicola* (Robert-

Seilaniantz et al. 2011) indicating that miR393 might play a different role depending on the pathogen lifestyle. MiR393 overexpression also increases glucosinolate levels at the cost of camalexin, the major phytoalexin in Arabidopsis, which then renders the miR393 overexpressor plants more susceptible to necrotrophs (Robert-Seilaniantz et al. 2011). It will then be of interest to investigate whether *dcl1a-Ac* plants respond in a different manner (e.g. susceptibility or resistance) to infection by pathogens other than *M. oryzae* and *F. fujikuroi*.

A role for miR398 in protection against oxidative stress has been reported in Arabidopsis and rice plants, and transgenic rice lines overexpressing miR398 have been shown to exhibit resistance to *M. oryzae* (Jagadeeswaran et al. 2009; Zhu et al. 2011; Li et al. 2014). Down-regulation of miR398 in *dcl1a-Ac* plants is then consistent with a phenotype of susceptibility to *M. oryzae* in these plants. Other studies in Arabidopsis, however, demonstrated that miR398 negatively regulates immune responses against bacterial pathogens (Jagadeeswaran et al. 2009; Li et al. 2010). Again, these findings indicate that a miRNA (e.g. miR393, miR398) might function as positive or negative regulator of immune responses depending on the host plant and/or the type of pathogen. How this might be achieved has yet to be determined.

On the other hand, it was previously reported that *DCL1* is required for the production of endogenous siRNAs other than miRNAs in Arabidopsis, such as those regulating *R* gene-mediated ETI in bacterial disease resistance (nat-siRNAATGB2; Katiyar-Agarwal et al. 2006). The production of certain long-siRNAs also appears to be DCL1-dependent (AtlsiRNA-1; Katiyar-Agarwal et al. 2007). Therefore, the possibility that overexpression of *DCL1a* might affect the production of other types of small RNAs which, in turn, might regulate rice defense responses should be considered. In other studies, Arabidopsis *dcl4* mutants, but not *dcl2* mutants, were found to be more susceptible to *Verticillium dahliae* (Ellendorff et al. 2009) further illustrating the relevance of small RNA pathway components in plant immunity.

Although a plethora of rice miRNAs have been shown to be regulated by pathogen infection in rice, the biological function remains largely unknown for most of them. Changes induced by *OsDCL1a* activation in the miRNAome are expected to cause alterations in the expression of their corresponding target genes. To better understand the impact of alterations in the miRNAome caused by *DCL1a* overexpression and how these alterations might contribute to disease resistance in rice, a better knowledge on target genes for rice miRNAs is a requisite. Clearly, alterations in *OsDCL1a* expression and accompanying alterations in miRNA levels, might affect diverse biological processes that are under miRNA regulation which might then decrease the plant's ability to cope with pathogen infection. The main challenge now is to elucidate how components of the miRNA biogenesis pathway and miRNAs themselves function in regulating mechanisms involved in disease resistance in rice. Understanding these mechanisms will provide powerful tools for the development of novel strategies to improve disease resistance in plants.

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References

Agrawal, G. K., Rakwal, R., Jwa, N. S., and Agrawal, 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. Biochem. 39:1095–1103

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

Bagnaresi, P., Biselli, C., Orrù, L., Urso, S., Crispino, L., Abbruscato, P., Piffanelli, P., Lupotto, E., Cattivelli, L., Valè, G. 2012. Comparative Transcriptome Profiling of the Early Response to Magnaporthe oryzae in Durable Resistant vs Susceptible Rice (Oryza sativa L.) Genotypes. PLoS One. 7:1–26

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

Bigeard, J., Colcombet, J., and Hirt, H. 2015. Signaling mechanisms in pattern-triggered immunity (PTI). Mol. Plant. 8:521–539

Bolger, A. M., Lohse, M., and Usadel, B. 2014. Genome analysis Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 30:2114–2120

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

Boyes, D. C., Nam, J., and Dangl, J. L. 1998. The Arabidopsis thaliana RPM1 disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. Proc. Natl. Acad. Sci. U. S. A. 95:15849–15854

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y. Y., Sieburth, L., and Voinnet, O. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. Science (80-.). 320:1185–1190

Camejo, D., Guzmán-Cedeño, Á., and Moreno, A. 2016. Reactive oxygen species, essential molecules, during plantepathogen interactions. Plant Physiol. Biochem. 103:10–23

Campo, S., Baldrich, P., Messeguer, J., Lalanne, E., Coca, M., and San Segundo, B. 2014. Overexpression of a Calcium-Dependent Protein Kinase Confers Salt and Drought Tolerance in Rice by Preventing Membrane Lipid Peroxidation. Plant Physiol. 165:688–704

Campo, S., Peris-Peris, C., Siré, C., Moreno, A. B., Donaire, L., Zytnicki, M., Notredame, C., Llave, C. and San Segundo, B. 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 Phytol. 6:212–227

Campos-Soriano, L., and Segundo, B. 2012. The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defence-related genes in rice leaves and confers resistance to pathogen infection. Mol. Plant Pathol. 13:579–592

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

Chuck, G., Meeley, R., Irish, E., Sakai, H., and Hake, S. 2007. The maize tasselseed4 microRNA controls sex determination and meristem cell fate by targeting Tasselseed6/indeterminate spikelet1. Nat. Genet. 39:1517–1521

Couto, D., and Zipfel, C. 2016. Regulation of pattern recognition receptor signalling in plants. Nat. Rev. Immunol. 16:537–552

Delteil, A., Gobbato, E., Cayrol, B., Estevan, J., Michel-Romiti, C., Dievart, A., Kroj, T., and Morel, J.-B. 2016. Several wall-associated kinases participate positively and negatively in basal defense against rice blast fungus. BMC Plant Biol. 16:17

Dillon, V. M., Overton, J., Grayer, R. J., and Harbone, Jeffrey B. 1997. Differences in phytoalexin response among rice cultivars of different resistance to blast. Phytochemistry. 44:599–603

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T. R. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 29:15–21

Du, Z., Zhou, X., Ling, Y., Zhang, Z., and Su, Z. 2010. agriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Res. 38:64–70

Ellendorff, U., Fradin, E. F., De Jonge, R., and Thomma, B. P. H. J. 2009. RNA silencing is required for Arabidopsis defence against Verticillium wilt disease. J. Exp. Bot. 60:591–602

Fei, Q., Xia, R., and Meyers, B. C. 2013. Phased, Secondary, Small Interfering RNAs in Posttranscriptional Regulatory Networks. Plant Cell. 25:2400–2415

Fei, Q., Zhang, Y., Xia, R., and Meyers, B. C. 2016. Small RNAs Add Zing to the Zig-Zag-Zig Model of Plant Defenses. Mol. Plant. Microbe. Interact. 29:165–9

Fradin, E. F., Zhang, Z., Juarez Ayala, J. C., Castroverde, C. D. M., Nazar, R. N., Robb, J., Liu, C.-M., and Thomma, B. P. H. J. 2009. Genetic dissection of Verticillium wilt resistance mediated by tomato Ve1. Plant Physiol. 150:320–32

Fujii, H., Chiou, T. J., Lin, S. I., Aung, K., and Zhu, J. K. 2005. A miRNA involved in phosphate-starvation response in Arabidopsis. Curr. Biol. 15:2038–2043

Goff, S. A., Ricke, D., Lan, T., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Varma, H., Hadley, D., Hutchison, D., Martin, C., Katagiri, F., Lange, B. M., Moughamer, T., Xia, Y., Budworth, P., Zhong, J., Miguel, T., Paszkowski, U., Zhang, S., Colbert, M., Sun, W., Chen, L., Cooper, B., Park, S., Wood, T. C., Mao, L., Quail, P., Wing, R., Dean, R., Yu, Y., Zharkikh, A., Shen, R., Sahasrabudhe, S., Thomas, A., Cannings, R., Gutin, A., Pruss, D., Reid, J., Tavtigian, S., Mitchell, J., Eldredge, G., Scholl, T., Miller, R. M., Bhatnagar, S., Adey, N., Rubano, T., Tusneem, N., Robinson, R., Feldhaus, J., Macalma, T., Oliphant, A., and Briggs, S. 2002. A Draft Sequence of the Rice Genome (Oryza sativa L. ssp. japonica). Science (80-.). 296:92–100

Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A., and Mans, J. 2000. The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J. 23:441–450

Hasegawa, M., Mitsuhara, I., Seo, S., Imai, T., Koga, J., Okada, K., Yamane, H., and Ohashi, Y. 2010. Phytoalexin Accumulation in the Interaction Between Rice and the Blast Fungus. Mol. Plant-Microbe Interact. 23:1000–1011

Hsing, Y., Lu, C., Fan, M., Sun, P., Lo, S., Lee, P., Ho, S., Lii, K., Wen Ko, H., Chen, S., Chung, S., Chen, J., Hour, C., Lin, Y., Chang, A., Wang, Y., Chen, Y., Lin, Y., Li, Y., Yen, H., Lai, C., Tseng, C., Chen, M., Huang, S., Yu, L., and Su-may. 2007. A rice gene activation / knockout mutant resource for high throughput functional genomics. Plan Mol. Biol. 63:351–364

Huang, J., Yang, M., Lu, L., and Zhang, X. 2016. Diverse Functions of Small RNAs in Different Plant – Pathogen Communications. Front. Microbiol. 7:1–17

Inoue, Y., Sakai, M., Yao, Q., Tanimoto, Y., Toshima, H., and Hasegawa, M. 2013. Identification of a Novel Casbane-Type Diterpene Phytoalexin, ent-10-Oxodepressin, from Rice Leaves. Biosci. Biotechnol. Biochem. 77:760–765

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

Jeon, Y.-A., Yu, S.-H., Lee, Y. Y., Park, H.-J., Lee, S., Sung, J. S., Kim, Y.-G., and Lee, H.-S. 2013. Incidence, Molecular Characteristics and Pathogenicity of Gibberella fujikuroi Species Complex Associated with Rice Seeds from Asian Countries. Mycobiology. 41:225–233

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

Jones-Rhoades, M. W., Bartel, D. P., and Bartel, B. 2006. MicroRNAS and their regulatory roles in plants. Annu. Rev. Plant Biol. 57:19–53

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

Kapoor, M., Arora, R., Lama, T., Nijhawan, A., Khurana, J. P., Tyagi, A. K., and Kapoor, S. 2008. Genome-wide identification, organization and phylogenetic analysis of Dicer-like, Argonaute and RNA-dependent RNA Polymerase gene families and their expression analysis during reproductive development and stress in rice. BMC Genomics. 9:451

Katiyar-Agarwal, S., Gao, S., Vivian-smith, A., Jin, H., Katiyar-agarwal, S., Gao, S., Vivian-smith, A., and Jin, H. 2007. A novel class of bacteria-induced small RNAs in Arabidopsis A novel class of bacteria-induced small RNAs in Arabidopsis. Genes Dev. 21:3123–3134

Katiyar-Agarwal, S., Morgan, R., Dahlbeck, D., Borsani, O., Villegas, A., Zhu, J.-K., Staskawicz, B. J., and Jin, H. 2006. A pathogen-inducible endogenous siRNA in plant immunity. Proc. Natl. Acad. Sci. U. S. A. 103:18002–7

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

Li, H., Zhou, S. Y., Zhao, W. S., Su, S. C., and Peng, Y. L. 2009. A novel wall-associated receptor-like protein kinase gene, OsWAK1, plays important roles in rice blast disease resistance. Plant Mol. Biol. 69:337–346

Li, Y., Lu, Y., Shi, Y., Wu, L., Xu, Y., Huang, F., Guo, X., Zhang, Y., Fan, J., Zhao, J., Zhang, H., Xu, P., Zhou, J., Wu, X., Wang, P., and Wang, W. 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., and Zhou, J.-M. 2010. Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. Plant Physiol. 152:2222–2231

Li, Y., Zhao, S.-L., Li, J.-L., Hu, X.-H., Wang, H., Cao, X.-L., Xu, Y.-J., Zhao, Z.-X., Xiao, Z.-Y., Yang, N., Fan, J., Huang, F., and Wang, W.-M. 2017. Osa-miR169 Negatively Regulates Rice Immunity against the Blast Fungus Magnaporthe oryzae. Front. Plant Sci. 8:1–13

Li, Z.-Y., Xia, J., Chen, Z., Yu, Y., Li, Q.-F., Zhang, Y.-C., Zhang, J.-P., Wang, C.-Y., Zhu, X.-Y., Zhang, W., and Chen, Y.-Q. 2016. Large-scale rewiring of innate immunity circuitry and microRNA regulation during initial rice blast infection. Sci. Rep. 6:25493

Liao, Y., Smyth, G. K., and Shi, W. 2014. Sequence analysis featureCounts : an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics. 30:923–930

Lichtenthaler, H. K., and Buschmann, C. 2001. Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. Current Protocols in Food Analytical Chemistry. F:F4:F4.3.

Liu, B., Li, P., Li, X., Liu, C., Cao, S., Chu, C., and Cao, X. 2005. Loss of Function of OsDCL1 Affects MicroRNA Accumulation and Causes Developmental. Plant Phys. 139:296–305

Llave, C., Kasschau, K. D., Rector, M. A., and Carrington, J. C. 2002. Endogenous and Silencing-Associated Small RNAs in Plants. Plant Cell. 14:1605–1619

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

Margis, R., Fusaro, A. F., Smith, N. A., Curtin, S. J., Watson, J. M., Finnegan, E. J., and Waterhouse, P. M. 2006. The evolution and diversification of Dicers in plants. FEBS Lett. 580:2442–2450

Meng, Y., Shao, C., Wang, H., and Chen, M. 2014. Uncovering DCL1-dependent small RNA loci on plant genomes: A structure-based approach. J. Exp. Bot. 65:395–400

Midoh, N., and Iwata, M. 1996. Cloning and characterization of a probenazole-inducible gene for an intracellular pathogenesis-related protein in rice. Plant Cell Physiol. 37:9–18

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J. D. G. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science (80-.). 312:436–439

Navarro, L., Jay, F., Nomura, K., He, S. Y., and Voinnet, O. 2008. Suppression of the microRNA pathway by bacterial effector proteins. Science (80-.). 321:964–967

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

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

Rau, A., Gallopin, M., Celeux, G., and Jaffrézic, F. 2013. Gene expression Data-based filtering for replicated high-throughput transcriptome sequencing experiments. Bioinformatics. 29:2146–2152

Robert-Seilaniantz, A., MacLean, D., Jikumaru, Y., Hill, L., Yamaguchi, S., Kamiya, Y., and Jones, J. D. G. 2011. The microRNA miR393 re-directs secondary metabolite biosynthesis away from camalexin and towards glucosinolates. Plant J. 67:218–231

Robinson, M. D., Mccarthy, D. J., and Smyth, G. K. 2010. edgeR : a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26:139–140

Schmelz, E. A., Huffaker, A., Sims, J. W., Christensen, S. A., Lu, X., Okada, K., and Peters, R. J. 2014. Biosynthesis, elicitation and roles of monocot terpenoid phytoalexins. Plant J. 79:659–678

Seo, J., Wu, J., Lii, Y., Li, Y., and Jin, H. 2013. Contribution of Small RNA Pathway Components in Plant Immunity. Mol. Plant-Microbe Interact. 26:617–625

Song, X., Li, P., Zhai, J., Zhou, M., Ma, L., Liu, B., Jeong, D.-H., Nakano, M., Cao, S., Liu, C., Chu, C., Wang, X.-J., Green, P. J., Meyers, B. C., and Cao, X. 2012. Roles of DCL4 and DCL3b in rice phased small RNA biogenesis. Plant J. 69:462–474

Umemura, K., Ogawa, N., Shimura, M., Koga, J., Usami, H., and Kono, T. 2003. Possible role of phytocassane, rice phytoalexin, in disease resistance of rice against the blast fungus Magnaporthe grisea. Biosci. Biotechnol. Biochem. 67:899–902

Van Loon, L. C., Rep, M., and Pieterse, C. M. J. 2006. Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. 44:135–62

Vaucheret, H., Mallory, A. C., and Bartel, D. P. 2006. AGO1 Homeostasis Entails Short Article Coexpression of MIR168 and AGO1 and Preferential Stabilization of miR168 by AGO1. Mol. Cell. 22:129–136

Wei, T., Ou, B., Li, J., Zhao, Y., Guo, D., Zhu, Y., Chen, Z., Gu, H., Li, C., Qin, G., and Qu, L. J. 2013. Transcriptional Profiling of Rice Early Response to

Magnaporthe oryzae Identified OsWRKYs as Important Regulators in Rice Blast Resistance. PLoS One. 8: e59720

Weiberg, A., Wang, M., Lin, F.-M., Zhao, H., Zhang, Z., Kaloshian, I., Huang, H.-D., Jin, H., Khaloshian, I., Huang, H.-D., and Jin, H. 2014. Fungal Small RNAs Suppress Plant Immunity by Hijacking Host RNA Interference Pathways. Science. 342:118–123

Wilson, R. A., and Talbot, N. J. 2009. Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. Nat. Rev. 7:185–195

Wulff, E. G., Sørensen, J. L., Lübeck, M., Nielsen, K. F., Thrane, U., and Torp, J. 2010. Fusarium spp. associated with rice Bakanae: Ecology, genetic diversity, pathogenicity and toxigenicity. Environ. Microbiol. 12:649–657

Xie, Z., Kasschau, K. D., and Carrington, J. C. 2003. Negative Feedback Regulation of Dicer-Like1 in Arabidopsis by microRNA-Guided mRNA Degradation. Curr. Biol. 13:784–789

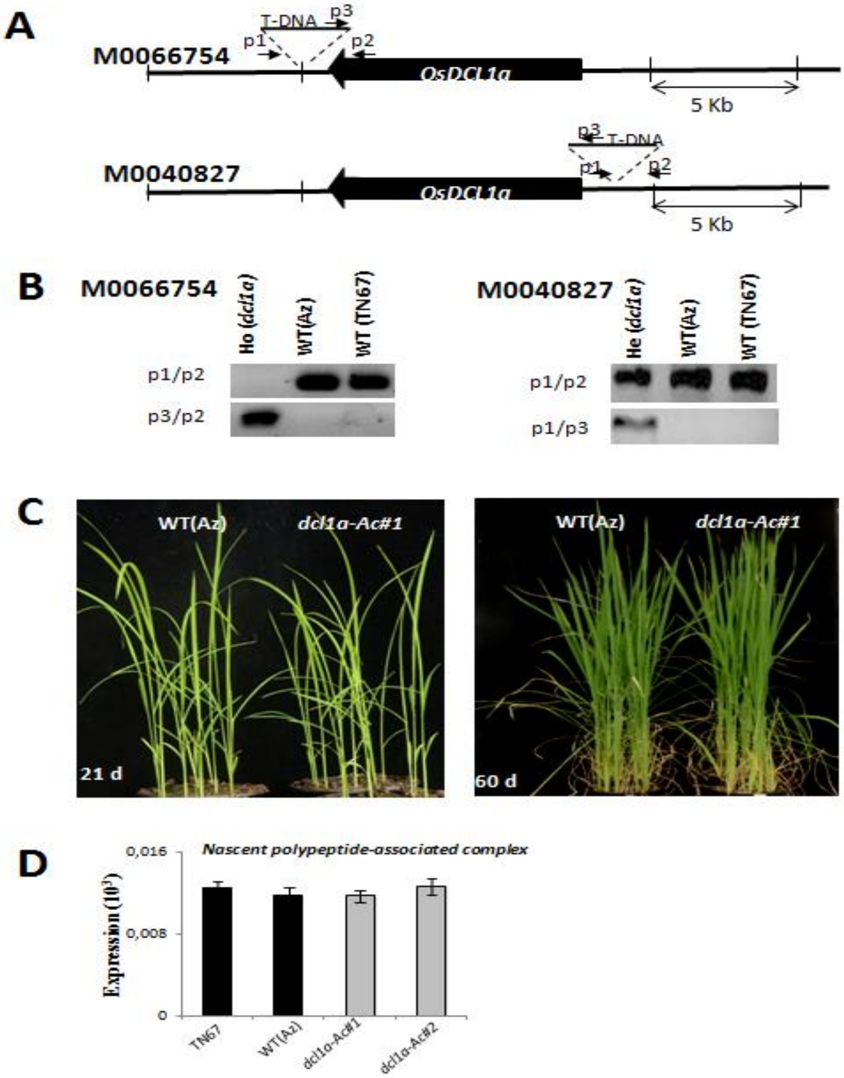
Yamane, H. 2013. Biosynthesis of Phytoalexins and Regulatory Mechanisms of It in Rice. Biosci. Biotechnol. Biochem. 77:1141–1148

Yu, J., Hu, S., Wang, J., Wong, G. K., Li, S., Liu, B., Deng, Y., Dai, L., Zhou, Y., Zhang, X., Cao, M., Liu, J., Sun, J., Tang, J., Chen, Y., Huang, X., Lin, W., Ye, C., Tong, W., Cong, L., Geng, J., Han, Y., Li, L., Li, W., Hu, G., Huang, X., Li, W., Li, J., Liu, Z., Li, L., Liu, J., Qi, Q., Liu, J., Li, L., Li, T., Wang, X., Lu, H., Wu, T., Zhu, M., Ni, P., Han, H., Dong, W., Ren, X., Feng, X., Cui, P., Li, X., Wang, H., Xu, X., Zhai, W., Xu, Z., Zhang, J., He, S., Zhang, J., Xu, J., Zhang, K., Zheng, X., Dong, J., Zeng, W., Tao, L., Ye, J., Tan, J., Ren, X., Chen, X., He, J., Liu, D., Tian, W., Tian, C., Xia, H., Bao, Q., Li, G., Gao, H., Cao, T., Wang, J., Zhao, W., Li, P., Chen, W., Wang, X., Zhang, Y., Hu, J., Wang, J., Liu, S., Yang, J., Zhang, G., Xiong, Y., Li, Z., Mao, L., Zhou, C., Zhu, Z., Chen, R., Hao, B., Zheng, W., Chen, S., and Guo, W. 2002. A Draft Sequence of the Rice Genome (Oryza sativa L. ssp. indica). Science (80-.). 296:79–93

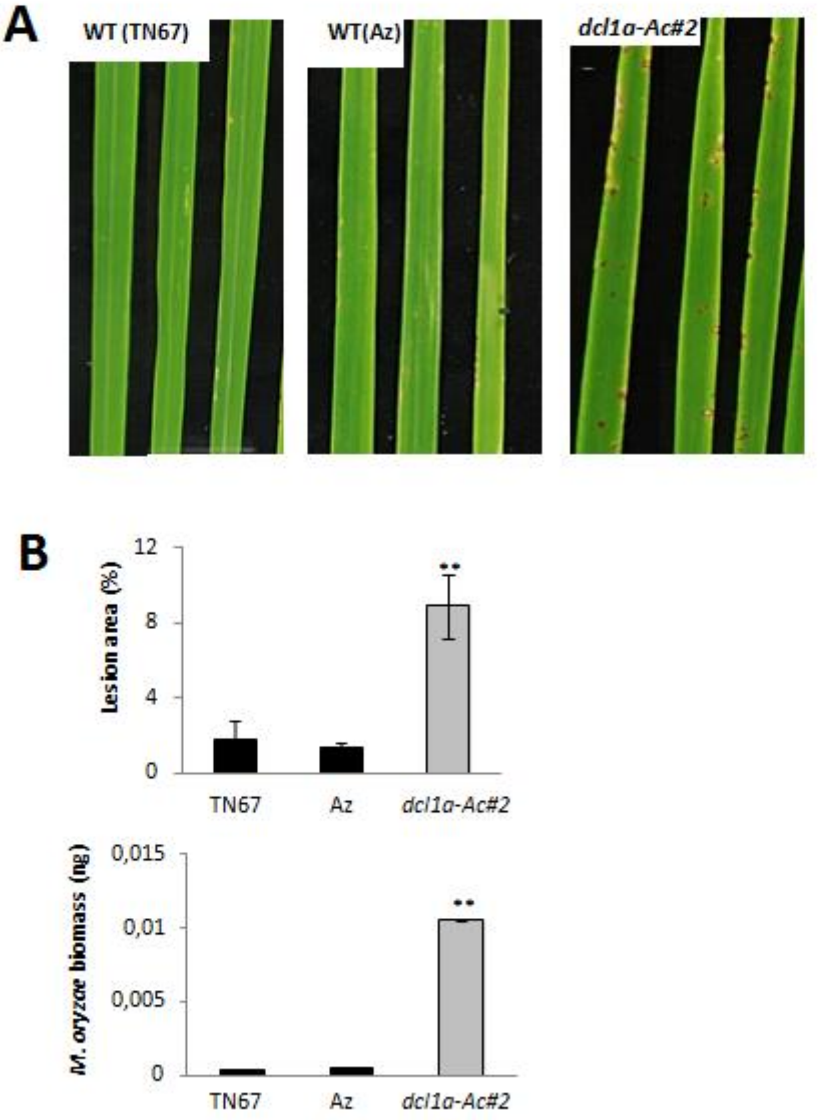
Zhang, D., Liu, M., Tang, M., Dong, B., Wu, D., Zhang, Z., and Zhou, B. 2015. Repression of microRNA biogenesis by silencing of OsDCL1 activates the basal resistance to Magnaporthe oryzae in rice. Plant Sci. 237:24–32

Zhu, C., Ding, Y., and Liu, H. 2011. MiR398 and plant stress responses. Physiol. Plant. 143:1–9

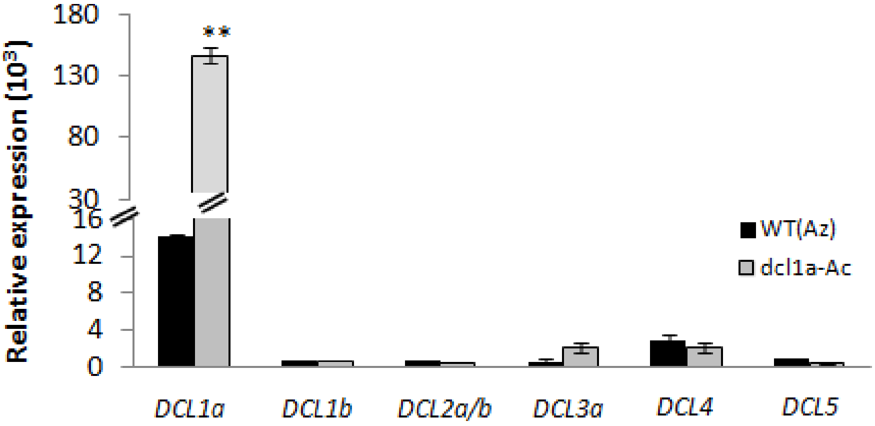
Supplemental Material



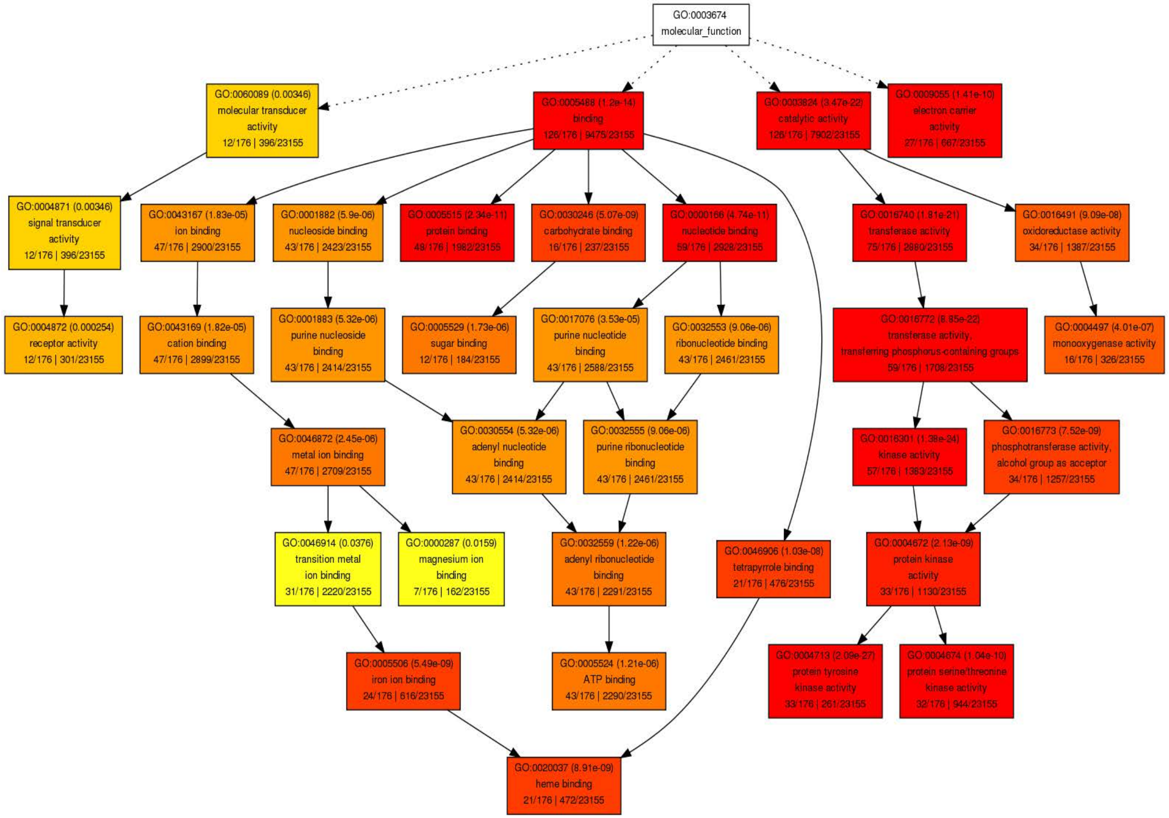
Supplemental Figure 1. Analysis of *OsDCL1a-Ac* mutant plants. A. Schematic representation of the T-DNA insertion rice mutants (TRIM collection, M0066754 and M0040827 mutants). The position of primers used for the analysis of T-DNA integration is shown (p1, p2, p3). **B.** PCR amplified DNA fragments for homozygous (Ho), hemizygous (He) and azygous (Az, segregated) plants. The nucleotide sequence of the amplification fragments was confirmed. **C.** Appearance of wild-type (segregated azygous plants) and *dcl1a-Ac* plants at the indicated developmental stages. **D.** Accumulation of *Nascent polypeptide-associated complex* transcripts in *dcl1a-Ac* plants. Differences were not statistically significant (ANOVA test).



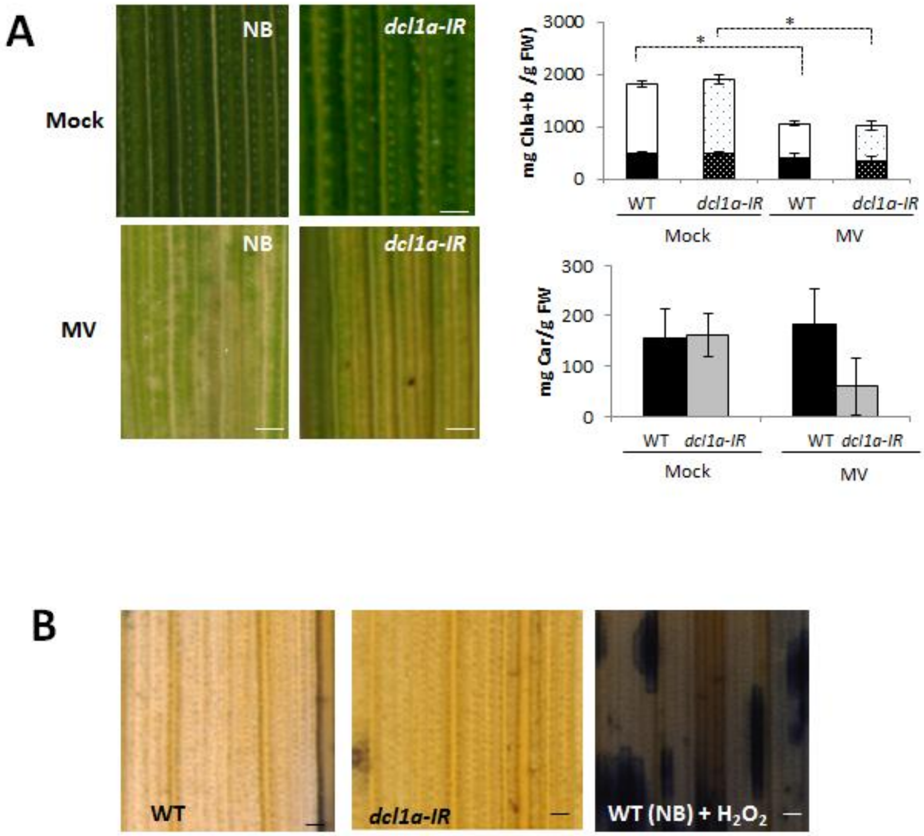
Supplemental Figure 2. Susceptibility of *dcl1a-Ac#2* plants to infection by the fungal pathogen *M. oryzae*. A. Disease symptoms at 7 dpi with *M. oryzae* spores (5 x 10^5 spores/mL). **B.** Lesion area of *M. oryzae*-infected leaves and quantification of *M. oryzae* DNA by qPCR, at 7 dpi. Statistical significance was determined by ANOVA (**, p-value ≤ 0.01).



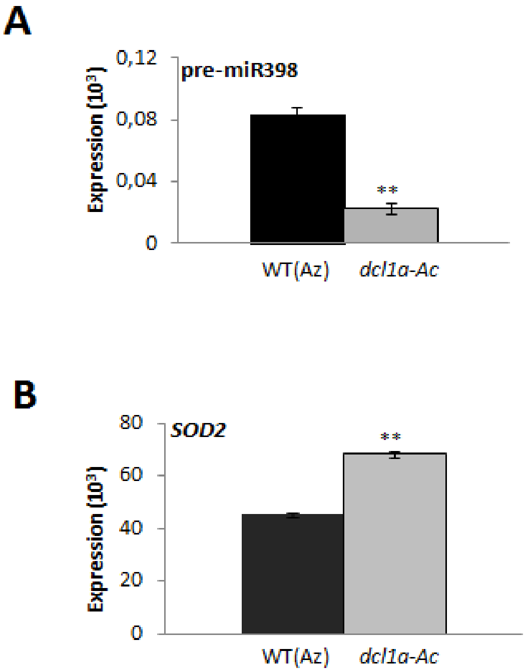
Supplemental Figure 3. Expression of *OsDCL* genes in wild-type and *dcl1a-Ac* plants grown under normal conditions (non-infected plants). The expression of *OsDCL1a* (Os03g02970), *OsDCL1b* (Os06g25250), *OsDCL2a/b* (Os03g38740/Os09g14610), *OsDCL3a* (Os01g68120), *OsDCL4* (Os04g43050) and *OsDCL5* (Os10g34430) was examined by RT-qPCR. Histograms show the mean ± SD of tree biological replicates, each with 12 plants per genotype. Statistical significance was determined by ANOVA (**, P ≤ 0.01). Except for *DCL1a*, no significant differences were observed in the expression of *OsDCL1* genes between wild type and *dcl1a-Ac* plants.



Supplemental Figure 4. Categorization by molecular function of up- and down-regulated genes in *dcl1a-Ac* mutant compared to wild-type (azygous) at control conditions. The gene onthology analysis was perform using AgriGO toolkit with the following parameter settings (i) Fisher's exact test with Bonferroni multiple comparison correction (ii) significance level α= 0.05 and (iii) using *Oryza sativa* NCBI database.

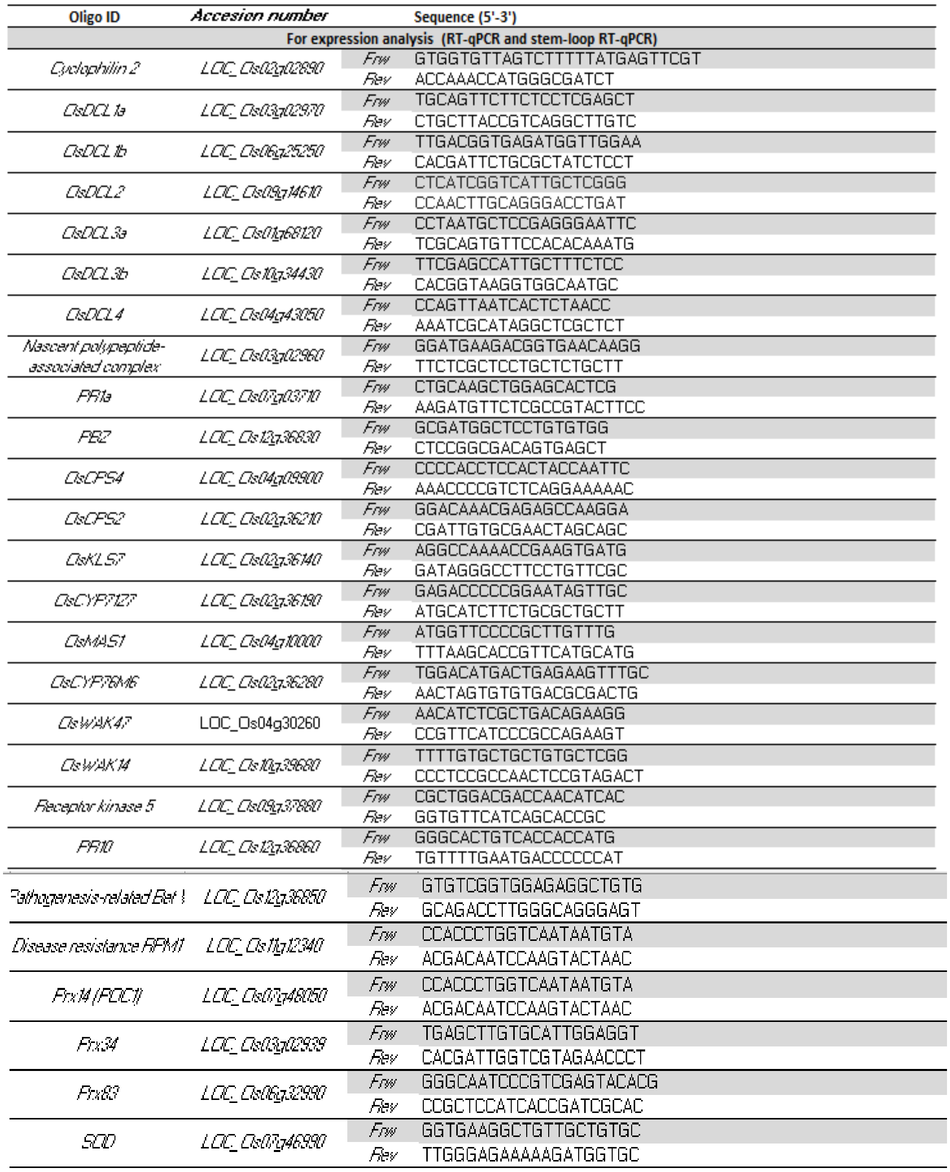


Supplemental Figure 5. Effect of treatment with methyl viologen (MV), chlorophyll and carotenoid content, and detection of superoxide ion ($O_2^{\bullet -}$) in knock-down *DCL11a* (*dcl1a-IR*; Nipponbare background) plants. A. Leaves of three-week old *dcl1a-IR* and wild-type (Nipponbare) plants were treated with MV for 72 h. Right panels: quantification of chorophyls (Chla + Chlb) and carotenoids (Car) in mock-inoculated and MV-treated wild-type and *dcl1a-IR* plants. Data shown correspond to wild-type (black, Chla; white Chlb) and *dcl1a-IR* (dotted) plants. Asterisks indicate significant differences (ANOVA test; *, P ≤ 0.05). **B.** Histochemical assays for detection of superoxide ion ($O_2^{\bullet -}$) by nitroblue tetrazolium (NBT) staining in leaves of wild-type and *dcl1a-IR* plants. As a control, leaves were treated with H_2O_2 for 6 h. Bars represent 250 µm.

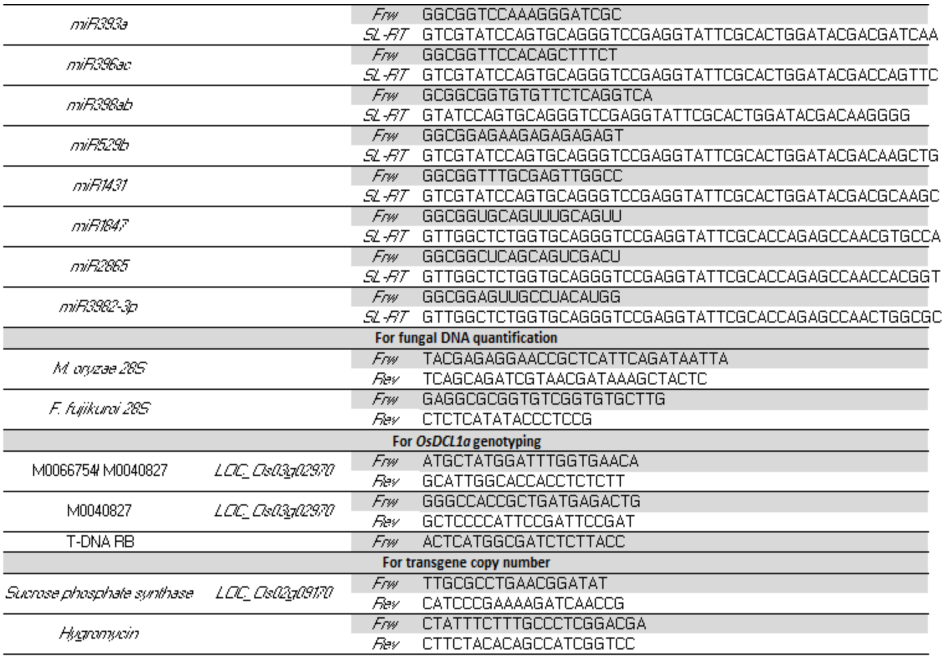


Supplemental Figure 6. Accumulation of miR398 precursor transcripts (pre-miR398) and miR398-targeted *OsSOD2* (Os07g46990) transcripts was determined by RT-qPCR. Asterisks indicate statistical significance (ANOVA test; **, P ≤ 0.01).

Supplemental Table 1. Sequences of oligonucleotides used.



Oligo ID	***Accesion number***		Sequence (5'-3')
			For expression analysis (RT-qPCR and stem-loop RT-qPCR)
Cyclophilin 2	*LOC_Os02g02890*	*Frw*	GTGGTGTTAGTCTTTTTATGAGTTCGT
		Rev	ACCAAACCATGGGCGATCT
OsDCL1a	*LOC_Os03g02970*	*Frw*	TGCAGTTCTTCTCCTCGAGCT
		Rev	CTGCTTACCGTCAGGCTTGTC
OsDCL1b	*LOC_Os06g25250*	*Frw*	TTGACGGTGAGATGGTTGGAA
		Rev	CACGATTCTGCGCTATCTCCT
OsDCL2	*LOC_Os09g14610*	*Frw*	CTCATCGGTCATTGCTCGGG
		Rev	CCAACTTGCAGGGACCTGAT
OsDCL3a	*LOC_Os01g68120*	*Frw*	CCTAATGCTCCGAGGGAATTC
		Rev	TCGCAGTGTTCCACACAAATG
OsDCL3b	*LOC_Os10g34430*	*Frw*	TTCGAGCCATTGCTTTCTCC
		Rev	CACGGTAAGGTGGCAATGC
OsDCL4	*LOC_Os04g43050*	*Frw*	CCAGTTAATCACTCTAACC
		Rev	AAATCGCATAGGCTCGCTCT
Nascent polypeptide-associated complex	*LOC_Os03g02960*	*Frw*	GGATGAAGACGGTGAACAAGG
		Rev	TTCTCGCTCCTGCTCTGCTT
PR1a	*LOC_Os07g03710*	*Frw*	CTGCAAGCTGGAGCACTCG
		Rev	AAGATGTTCTCGCCGTACTTCC
PBZ	*LOC_Os12g36830*	*Frw*	GCGATGGCTCCTGTGTGG
		Rev	CTCCGGCGACAGTGAGCT
OsCPS4	*LOC_Os04g09900*	*Frw*	CCCCACCTCCACTACCAATTC
		Rev	AAACCCCGTCTCAGGAAAAAC
OsCPS2	*LOC_Os02g36210*	*Frw*	GGACAAACGAGAGCCAAGGA
		Rev	CGATTGTGCGAACTAGCAGC
OsKLS7	*LOC_Os02g36140*	*Frw*	AGGCCAAAACCGAAGTGATG
		Rev	GATAGGGCCTTCCTGTTCGC
OsCYP71Z7	*LOC_Os02g36190*	*Frw*	GAGACCCCCGGAATAGTTGC
		Rev	ATGCATCTTCTGCGCTGCTT
OsMAS1	*LOC_Os04g10000*	*Frw*	ATGGTTCCCCGCTTGTTTG
		Rev	TTTAAGCACCGTTCATGCATG
OsCYP76M6	*LOC_Os02g36280*	*Frw*	TGGACATGACTGAGAAGTTTGC
		Rev	AACTAGTGTGTGACGCGACTG
OsWAK47	LOC_Os04g30260	*Frw*	AACATCTCGCTGACAGAAGG
		Rev	CCGTTCATCCCGCCAGAAGT
OsWAK14	*LOC_Os10g39680*	*Frw*	TTTTGTGCTGCTGTGCTCGG
		Rev	CCCTCCGCCAACTCCGTAGACT
Receptor kinase 5	*LOC_Os09g37880*	*Frw*	CGCTGGACGACCAACATCAC
		Rev	GGTGTTCATCAGCACCGC
PR10	*LOC_Os12g36860*	*Frw*	GGGCACTGTCACCACCATG
		Rev	TGTTTTGAATGACCCCCCAT
Pathogenesis-related Bet 1	*LOC_Os12g36850*	*Frw*	GTGTCGGTGGAGAGGCTGTG
		Rev	GCAGACCTTGGGCAGGGAGT
Disease resistance RPM1	*LOC_Os11g12340*	*Frw*	CCACCCTGGTCAATAATGTA
		Rev	ACGACAATCCAAGTACTAAC
Prx14 (POC1)	*LOC_Os07g48050*	*Frw*	CCACCCTGGTCAATAATGTA
		Rev	ACGACAATCCAAGTACTAAC
Prx34	*LOC_Os03g02939*	*Frw*	TGAGCTTGTGCATTGGAGGT
		Rev	CACGATTGGTCGTAGAACCCT
Prx83	*LOC_Os06g32990*	*Frw*	GGGCAATCCCGTCGAGTACACG
		Rev	CCGCTCCATCACCGATCGCAC
SOD	*LOC_Os07g46990*	*Frw*	GGTGAAGGCTGTTGCTGTGC
		Rev	TTGGGAGAAAAAGATGGTGC



miR393a		*Frw*	GGCGGTCCAAAGGGATCGC
		SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATCAA
miR396ac		*Frw*	GGCGGTTCCACAGCTTTCT
		SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGTTC
miR398ab		*Frw*	GCGGCGGTGTGTTCTCAGGTCA
		SL-RT	GTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG
miR529b		*Frw*	GGCGGAGAAGAGAGAGAGT
		SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGCTG
miR1431		*Frw*	GGCGGTTTGCGAGTTGGCC
		SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCAAGC
miR1847		*Frw*	GGCGGUGCAGUUUGCAGUU
		SL-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGTGCCA
miR2865		*Frw*	GGCGGCUCAGCAGUCGACU
		SL-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCACGGT
miR3982-3p		*Frw*	GGCGGAGUUGCCUACAUGG
		SL-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTGGCGC
For fungal DNA quantification			
M. oryzae 28S		*Frw*	TACGAGAGGAACCGCTCATTCAGATAATTA
		Rev	TCAGCAGATCGTAACGATAAAGCTACTC
F. fujikuroi 28S		*Frw*	GAGGCGCGGTGTCGGTGTGCTTG
		Rev	CTCTCATATACCCTCCG
For *OsDCL1a* genotyping			
M0066754/ M0040827	*LOC_Os03g02970*	*Frw*	ATGCTATGGATTTGGTGAACA
		Rev	GCATTGGCACCACCTCTCTT
M0040827	*LOC_Os03g02970*	*Frw*	GGGCCACCGCTGATGAGACTG
		Rev	GCTCCCCATTCCGATTCCGAT
T-DNA RB		*Frw*	ACTCATGGCGATCTCTTACC
For transgene copy number			
Sucrose phosphate synthase	*LOC_Os02g09170*	*Frw*	TTGCGCCTGAACGGATAT
		Rev	CATCCCGAAAAGATCAACCG
Hygromycin		*Frw*	CTATTTCTTTGCCCTCGGACGA
		Rev	CTTCTACACAGCCATCGGTCC

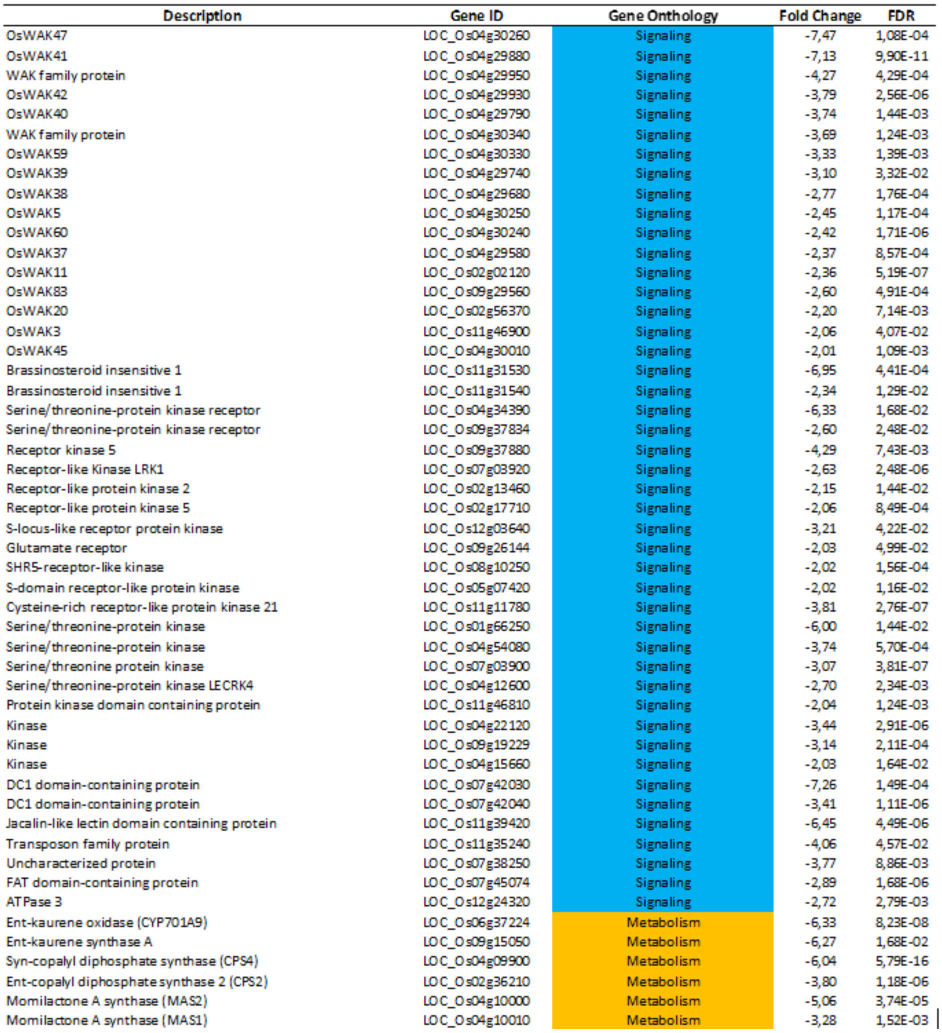
Supplemental Table 2. Determination of T-DNA copy number in *dcl1a-Ac* mutants by qPCR using the *sucrose phosphate synthase* (*SPS*) as endogenous reference gene.

	Number of copies
M0066754	0,988
M0040827	0,996

Supplemental Table 3. Statistics of RNA-seq in *dcl1a-Ac* and wild-type plants obtained from Solexa/Illumina sequencing datasets.

	wild-type	***dcl1a-Ac***
Raw reads	39,6 M	31,0 M
Quality and length triming	39,4 M	30,8 M
Genome mapping	39 M	30,3 M
t/r/sn/snoRNA reads	0,11 M	0,44 M
Unique mapped reads	37,6 M	28,5 M

Supplemental Table 4. Down-regulated genes in *dcl1a-Ac* plants relative to wild-type plants sorted by functional category.



Description	Gene ID	Gene Onthology	Fold Change	FDR
OsWAK47	LOC_Os04g30260	Signaling	-7,47	1,08E-04
OsWAK41	LOC_Os04g29880	Signaling	-7,13	9,90E-11
WAK family protein	LOC_Os04g29950	Signaling	-4,27	4,29E-04
OsWAK42	LOC_Os04g29930	Signaling	-3,79	2,56E-06
OsWAK40	LOC_Os04g29790	Signaling	-3,74	1,44E-03
WAK family protein	LOC_Os04g30340	Signaling	-3,69	1,24E-03
OsWAK59	LOC_Os04g30330	Signaling	-3,33	1,39E-03
OsWAK39	LOC_Os04g29740	Signaling	-3,10	3,32E-02
OsWAK38	LOC_Os04g29680	Signaling	-2,77	1,76E-04
OsWAK5	LOC_Os04g30250	Signaling	-2,45	1,17E-04
OsWAK60	LOC_Os04g30240	Signaling	-2,42	1,71E-06
OsWAK37	LOC_Os04g29580	Signaling	-2,37	8,57E-04
OsWAK11	LOC_Os02g02120	Signaling	-2,36	5,19E-07
OsWAK83	LOC_Os09g29560	Signaling	-2,60	4,91E-04
OsWAK20	LOC_Os02g56370	Signaling	-2,20	7,14E-03
OsWAK3	LOC_Os11g46900	Signaling	-2,06	4,07E-02
OsWAK45	LOC_Os04g30010	Signaling	-2,01	1,09E-03
Brassinosteroid insensitive 1	LOC_Os11g31530	Signaling	-6,95	4,41E-04
Brassinosteroid insensitive 1	LOC_Os11g31540	Signaling	-2,34	1,29E-02
Serine/threonine-protein kinase receptor	LOC_Os04g34390	Signaling	-6,33	1,68E-02
Serine/threonine-protein kinase receptor	LOC_Os09g37834	Signaling	-2,60	2,48E-02
Receptor kinase 5	LOC_Os09g37880	Signaling	-4,29	7,43E-03
Receptor-like Kinase LRK1	LOC_Os07g03920	Signaling	-2,63	2,48E-06
Receptor-like protein kinase 2	LOC_Os02g13460	Signaling	-2,15	1,44E-02
Receptor-like protein kinase 5	LOC_Os02g17710	Signaling	-2,06	8,49E-04
S-locus-like receptor protein kinase	LOC_Os12g03640	Signaling	-3,21	4,22E-02
Glutamate receptor	LOC_Os09g26144	Signaling	-2,03	4,99E-02
SHR5-receptor-like kinase	LOC_Os08g10250	Signaling	-2,02	1,56E-04
S-domain receptor-like protein kinase	LOC_Os05g07420	Signaling	-2,02	1,16E-02
Cysteine-rich receptor-like protein kinase 21	LOC_Os11g11780	Signaling	-3,81	2,76E-07
Serine/threonine-protein kinase	LOC_Os01g66250	Signaling	-6,00	1,44E-02
Serine/threonine-protein kinase	LOC_Os04g54080	Signaling	-3,74	5,70E-04
Serine/threonine protein kinase	LOC_Os07g03900	Signaling	-3,07	3,81E-07
Serine/threonine-protein kinase LECRK4	LOC_Os04g12600	Signaling	-2,70	2,34E-03
Protein kinase domain containing protein	LOC_Os11g46810	Signaling	-2,04	1,24E-03
Kinase	LOC_Os04g22120	Signaling	-3,44	2,91E-06
Kinase	LOC_Os09g19229	Signaling	-3,14	2,11E-04
Kinase	LOC_Os04g15660	Signaling	-2,03	1,64E-02
DC1 domain-containing protein	LOC_Os07g42030	Signaling	-7,26	1,49E-04
DC1 domain-containing protein	LOC_Os07g42040	Signaling	-3,41	1,11E-06
Jacalin-like lectin domain containing protein	LOC_Os11g39420	Signaling	-6,45	4,49E-06
Transposon family protein	LOC_Os11g35240	Signaling	-4,06	4,57E-02
Uncharacterized protein	LOC_Os07g38250	Signaling	-3,77	8,86E-03
FAT domain-containing protein	LOC_Os07g45074	Signaling	-2,89	1,68E-06
ATPase 3	LOC_Os12g24320	Signaling	-2,72	2,79E-03
Ent-kaurene oxidase (CYP701A9)	LOC_Os06g37224	Metabolism	-6,33	8,23E-08
Ent-kaurene synthase A	LOC_Os09g15050	Metabolism	-6,27	1,68E-02
Syn-copalyl diphosphate synthase (CPS4)	LOC_Os04g09900	Metabolism	-6,04	5,79E-16
Ent-copalyl diphosphate synthase 2 (CPS2)	LOC_Os02g36210	Metabolism	-3,80	1,18E-06
Momilactone A synthase (MAS2)	LOC_Os04g10000	Metabolism	-5,06	3,74E-05
Momilactone A synthase (MAS1)	LOC_Os04g10010	Metabolism	-3,28	1,52E-03

9-beta-pimara-7,15-diene oxidase (CYP93A3)	LOC_Os04g09920	Metabolism	-5,04	9,90E-11
Oryzalexin D synthase (CYP76M8)	LOC_Os02g36070	Metabolism	-4,97	9,46E-07
Isoflavone reductase	LOC_Os01g13610	Metabolism	-4,74	3,31E-06
Stemar-13-ene synthase (KSL8)	LOC_Os11g28530	Metabolism	-4,61	5,97E-08
Ent-sandaracopimara-8(14),15-diene synthase (KSL10)	LOC_Os12g30824	Metabolism	-4,11	1,07E-03
Terpene synthase (KSL7)	LOC_Os02g36140	Metabolism	-3,74	3,59E-06
Ent-cassadiene C2-hydroxylase (CYP71Z7)	LOC_Os02g36190	Metabolism	-4,18	4,08E-10
Oryzalexin E synthase (CYP76M6)	LOC_Os02g36280	Metabolism	-3,84	1,19E-07
Terpene synthase	LOC_Os04g27190	Metabolism	-2,42	7,54E-04
Ent-sandaracopimaradiene 3-hydroxylase (CYP701A8)	LOC_Os06g37300	Metabolism	-2,34	1,60E-03
Cytochrome P450	LOC_Os07g29960	Metabolism	-5,57	3,09E-08
Cytochrome P450 99A2 (CYP99A2)	LOC_Os04g10160	Metabolism	-4,21	1,54E-05
Cytochrome P450 76M5 (CYP76M5)	LOC_Os02g36030	Metabolism	-3,72	1,22E-02
Cytochrome P450	LOC_Os04g27020	Metabolism	-3,49	6,06E-03
Cytochrome P450	LOC_Os03g39690	Metabolism	-2,72	5,53E-03
Delta 12 oleic acid desaturase	LOC_Os07g23430	Metabolism	-9,28	9,31E-09
Fatty acid desaturase	LOC_Os07g23410	Metabolism	-7,26	4,74E-04
Agmatine hydroxycinnamoyltransferase 1	LOC_Os04g56910	Metabolism	-6,51	4,59E-09
SAM dependent carboxyl methyltransferase	LOC_Os06g20790	Metabolism	-5,82	4,17E-09
Glycine-rich RNA-binding, abscisic acid-inducible protein	LOC_Os04g55980	Metabolism	-4,51	2,98E-03
Laccase6	LOC_Os01g63180	Metabolism	-4,13	1,41E-05
Caffeate O-methyltransferase-like protein 2	LOC_Os04g01470	Metabolism	-2,84	2,32E-02
Acyl transferase 5	LOC_Os05g19910	Metabolism	-2,43	1,12E-03
HVA22	LOC_Os11g05800	Metabolism	-2,11	5,39E-03
Strictosidine synthase	LOC_Os10g39710	Metabolism	-2,06	2,50E-02
Verticillium wilt disease resistance protein	LOC_Os12g11860	Biotic stress	-8,58	5,26E-07
Verticillium wilt disease resistance protein	LOC_Os12g12130	Biotic stress	-3,63	6,14E-04
Disease resistance protein RPM1	LOC_Os11g12340	Biotic stress	-2,59	1,36E-05
Disease resistance protein RPM1	LOC_Os11g12320	Biotic stress	-2,54	1,85E-02
Leucine Rich Repeat family protein	LOC_Os12g11510	Biotic stress	-4,30	1,54E-02
Leucine Rich Repeat family protein	LOC_Os12g11940	Biotic stress	-4,06	3,50E-02
Leucine Rich Repeat family protein	LOC_Os11g35450	Biotic stress	-3,56	2,18E-02
Leucine-rich repeat family protein	LOC_Os10g05570	Biotic stress	-3,18	3,50E-02
Leucine Rich Repeat family protein	LOC_Os11g35840	Biotic stress	-2,73	3,88E-02
NBS-LRR protein	LOC_Os07g02610	Biotic stress	-4,15	4,99E-02
NBS-LRR-like protein	LOC_Os05g41310	Biotic stress	-3,04	8,37E-03
PR10	LOC_Os12g36860	Biotic stress	-4,55	3,05E-05
PR10	LOC_Os12g36840	Biotic stress	-3,49	3,32E-02
Pathogenesis-related Bet V (PR10)	LOC_Os12g36850	Biotic stress	-3,58	4,60E-10
Pathogenesis-related Bet V (PR10)	LOC_Os12g36880	Biotic stress	-2,45	2,11E-04
PR1b	LOC_Os01g28450	Biotic stress	-3,66	3,85E-08
PR1a	LOC_Os07g03710	Biotic stress	-3,15	8,53E-08
Transferase family protein	LOC_Os05g04930	Biotic stress	-6,58	2,43E-03
Uncharacterized protein	LOC_Os07g03730	Biotic stress	-3,92	1,88E-04
Calmodulin binding protein	LOC_Os11g44630	Biotic stress	-3,68	5,34E-04
NB-ARC domain containing protein	LOC_Os07g02570	Biotic stress	-2,47	5,00E-02
WRKY47	LOC_Os07g48260	Biotic stress	-2,39	6,59E-03
Cytochrome P450	LOC_Os06g43384	Oxidative stress	-5,72	5,00E-02
Cytochrome P450	LOC_Os02g36110	Oxidative stress	-3,55	2,63E-06
Cytochrome P450 72A21	LOC_Os01g43750	Oxidative stress	-2,30	5,87E-03
Peroxidase	LOC_Os05g04490	Oxidative stress	-3,16	2,76E-02
Peroxidase83 (Prx83)	LOC_Os06g32990	Oxidative stress	-2,78	4,05E-03
Glutathione S-transferase GSTU6	LOC_Os10g38600	Oxidative stress	-2,02	1,71E-02
OsbHLH025	LOC_Os01g09990	RNA processing and transcription	-5,28	1,05E-05
RNA-binding protein FUS	LOC_Os01g68730	RNA processing and transcription	-4,63	8,07E-04
Myb51 protein	LOC_Os08g33150	RNA processing and transcription	-2,18	2,06E-04

OsFBX327	LOC_Os09g21796	RNA processing and transcription	-2,06	1,17E-02
Ubiquitin family protein	LOC_Os11g18670	Protein synthesis and degradation	-6,12	3,12E-02
Carboxyl-terminal peptidase	LOC_Os01g37000	Protein synthesis and degradation	-5,66	1,68E-02
Uncharacterized protein	LOC_Os04g03100	Protein synthesis and degradation	-3,68	1,35E-02
OsSub13	LOC_Os02g16940	Protein synthesis and degradation	-3,30	1,09E-02
Subtilisin-like serine protease	LOC_Os01g58280	Protein synthesis and degradation	-2,90	1,42E-08
Midasin	LOC_Os10g31864	Protein synthesis and degradation	-2,15	8,57E-04
Flotillin-like protein 1	LOC_Os10g34040	Abiotic stress	-4,37	1,63E-04
Germin-like protein 8-10	LOC_Os08g09060	Abiotic stress	-2,25	2,20E-02
Purine permease	LOC_Os03g08880	Transport	-3,83	8,09E-04
Ammonium transporter 3 member 3	LOC_Os02g34580	Transport	-2,94	1,54E-02
H+-pyrophosphatase	LOC_Os01g23580	Transport	-2,67	1,28E-02
No apical meristem protein	LOC_Os07g04560	Development	-2,97	9,44E-04
Keratin, type I cytoskeletal 9	LOC_Os01g68720	DNA repair and cell cycle	-2,80	1,24E-03
keratin, type I cytoskeletal 9	LOC_Os01g68740	DNA repair and cell cycle	-2,28	1,76E-04
Chromatin remodeling protein-like	LOC_Os06g14406	DNA repair and cell cycle	-2,80	2,03E-04
Transposon family protein	LOC_Os04g03450	Unknown	-3,19	4,44E-02
Retrotransposon protein	LOC_Os03g19600	Unknown	-3,05	5,53E-03
Zinc finger protein	LOC_Os02g36000	Unknown	-2,73	9,71E-03
Retrotransposon	LOC_Os04g22080	Unknown	-2,67	3,74E-04
Alcohol dehydrogenase-like protein	LOC_Os07g46846	Unknown	-2,52	8,77E-03
Uncharacterized protein	LOC_Os01g33869	Unknown	-10,40	2,19E-10
Uncharacterized protein	LOC_Os06g42060	Unknown	-6,58	1,87E-03
Uncharacterized protein	LOC_Os11g45510	Unknown	-6,12	3,88E-02
Uncharacterized protein	LOC_Os04g39320	Unknown	-4,84	1,07E-07
Uncharacterized protein	LOC_Os09g19350	Unknown	-4,31	4,39E-03
Uncharacterized protein	LOC_Os09g13400	Unknown	-4,26	1,16E-02
Uncharacterized protein	LOC_Os08g29040	Unknown	-4,19	3,40E-02
Uncharacterized protein	LOC_Os06g45970	Unknown	-4,19	1,09E-02
Uncharacterized protein	LOC_Os08g25839	Unknown	-4,13	9,50E-04
Uncharacterized protein	LOC_Os08g26350	Unknown	-3,55	6,28E-03
Uncharacterized protein	LOC_Os12g11550	Unknown	-3,09	9,70E-03
Uncharacterized protein	LOC_Os02g16490	Unknown	-3,08	5,55E-03
Uncharacterized protein	LOC_Os06g46160	Unknown	-2,96	2,11E-02
Uncharacterized protein	LOC_Os12g41124	Unknown	-2,92	3,94E-04
Uncharacterized protein	LOC_Os09g13440	Unknown	-2,88	1,42E-03
Uncharacterized protein	LOC_Os02g27130	Unknown	-2,87	7,74E-03
Uncharacterized protein	LOC_Os08g02354	Unknown	-2,77	4,78E-04
Uncharacterized protein	LOC_Os08g26230	Unknown	-2,71	9,46E-07
Uncharacterized protein	LOC_Os09g21802	Unknown	-2,67	5,69E-03
Uncharacterized protein	LOC_Os01g48620	Unknown	-2,49	1,25E-04
Uncharacterized protein	LOC_Os12g21940	Unknown	-2,47	5,90E-04
Uncharacterized protein	LOC_Os11g29210	Unknown	-2,46	1,56E-02
Uncharacterized protein	LOC_Os08g08580	Unknown	-2,44	3,28E-02
Uncharacterized protein	LOC_Os08g10244	Unknown	-2,40	1,07E-06
Uncharacterized protein	LOC_Os08g39330	Unknown	-2,38	4,80E-03
Uncharacterized protein	LOC_Os04g58734	Unknown	-2,35	7,05E-03
Uncharacterized protein	LOC_Os03g03724	Unknown	-2,33	2,80E-03
Uncharacterized protein	LOC_Os11g37210	Unknown	-2,27	3,15E-02
Uncharacterized protein	LOC_Os09g16780	Unknown	-2,20	2,59E-02
Uncharacterized protein	LOC_Os01g29220	Unknown	-2,12	3,89E-02
Uncharacterized protein	LOC_Os06g44034	Unknown	-2,10	1,68E-02
Uncharacterized protein	LOC_Os02g06725	Unknown	-2,09	4,41E-04
Uncharacterized protein	LOC_Os03g18264	Unknown	-2,01	4,32E-06

Supplemental Table 5. Up-regulated genes in *dcl1a-Ac* plants relative to wild-type plants sorted by functional category.

Description	Gene ID	Gene Onthology	Fold Change	FDR
Phytosulfokine receptor	LOC_Os02g06090	Signaling	3,05	2,68E-03
Putative serine/threonine protein kinase	LOC_Os03g02980	Signaling	2,71	1,26E-02
Alpha-amylase	LOC_Os02g52710	Metabolism	7,04	1,54E-04
Inositol-tetrakisphosphate 1-kinase 4	LOC_Os02g26720	Metabolism	4,40	1,71E-02
Oleosin 16 kDa	LOC_Os04g46200	Metabolism	4,24	2,11E-04
Linoleate 9S-lipoxygenase 1	LOC_Os03g49260	Metabolism	4,22	1,14E-02
Linoleate 9S-lipoxygenase 2	LOC_Os03g52860	Metabolism	4,10	9,46E-07
Glycosyltransferase	LOC_Os09g34250	Metabolism	3,46	9,49E-06
Glycosyltransferase	LOC_Os04g47720	Metabolism	2,40	7,14E-03
Quercetin 3-O-glucoside-6''-O-malonyltransferase	LOC_Os02g28220	Metabolism	2,79	7,08E-04
Polygalacturonase inhibitor	LOC_Os05g01444	Metabolism	2,70	9,50E-04
Dihydroflavonol-4-reductase	LOC_Os06g46740	Metabolism	2,60	4,97E-03
Putrescine hydroxycinnamoyltransferase	LOC_Os09g37200	Metabolism	2,39	1,44E-02
Glucosyl transferase	LOC_Os03g55030	Metabolism	2,13	3,66E-02
Peroxidase34 (Prx34)	LOC_Os03g02939	Oxidative stress	5,63	9,49E-06
Peroxidase14 (Prx14) - POC1	LOC_Os07g48050	Oxidative stress	2,88	1,16E-02
Peroxidase110 (Prx110)	LOC_Os07g48010	Oxidative stress	2,16	4,58E-03
Cytochrome P450	LOC_Os09g10340	Oxidative stress	3,21	5,20E-03
Cytochrome P450 51	LOC_Os05g12040	Oxidative stress	2,87	2,96E-05
OsDCL1a	LOC_Os03g02970	RNA processing and transcription	3,20	4,55E-04
Protein TIFY 11e	LOC_Os10g25230	RNA processing and transcription	3,16	2,60E-02
Helix-loop-helix DNA-binding domain containing protein	LOC_Os03g55220	RNA processing and transcription	2,86	1,71E-03
Retrotransposon Ty1-copia	LOC_Os12g02470	RNA processing and transcription	2,27	2,80E-07
rRNA N-glycosidase	LOC_Os01g06740	RNA processing and transcription	2,18	4,88E-02
Uncharacterized protein	LOC_Os06g38080	RNA processing and transcription	2,17	1,71E-03
Ubiquitin family protein	LOC_Os05g38310	Protein synthesis and degradation	5,77	6,85E-03
Bowman-Birk type bran trypsin inhibitor	LOC_Os01g03680	Protein synthesis and degradation	2,54	6,14E-04
Germin-like protein 3-4	LOC_Os03g48760	Abiotic stress	5,92	9,80E-03
Germin-like protein 3-3	LOC_Os03g48750	Abiotic stress	3,44	1,72E-02
Late embryogenesis abundant protein 3	LOC_Os05g46480	Abiotic stress	2,84	2,15E-06
Rab 21	LOC_Os11g26790	Abiotic stress	2,79	1,17E-06
ABC transporter G family member 35	LOC_Os01g42370	Abiotic stress	2,71	4,09E-02
Terminal flower1	LOC_Os01g10590	Development	6,45	2,96E-03
Sex determination protein tasselseed-2	LOC_Os07g46920	Development	2,18	2,67E-03
Polygalacturonase inhibitor	LOC_Os05g01380	Cell wall	4,13	8,32E-06
Myb family protein-like	LOC_Os08g12950	Unknown	5,29	1,81E-02
Polyamine oxidase	LOC_Os09g20260	Unknown	4,93	1,92E-03
Subtilisin-chymotrypsin inhibitor CI-1	LOC_Os12g36240	Unknown	3,70	1,12E-03
Subtilisin-chymotrypsin inhibitor CI-1B	LOC_Os12g36210	Unknown	3,53	5,02E-03
Subitilisin-chymotrypsin inhibitor	LOC_Os12g25090	Unknown	2,63	2,14E-02
Late embryogenesis abundant protein	LOC_Os03g07180	Unknown	3,50	1,11E-03
Arginine decarboxylase 2	LOC_Os04g01690	Unknown	2,70	4,97E-03
Uncharacterized protein	LOC_Os03g02470	Unknown	10,17	6,97E-06
Uncharacterized protein	LOC_Os04g37890	Unknown	7,86	2,38E-05
Uncharacterized protein	LOC_Os06g14870	Unknown	6,02	6,14E-04
Uncharacterized protein	LOC_Os02g10960	Unknown	6,00	2,82E-02
Uncharacterized protein	LOC_Os07g34280	Unknown	4,25	2,80E-04
Uncharacterized protein	LOC_Os04g42050	Unknown	3,76	4,84E-02
Uncharacterized protein	LOC_Os07g02880	Unknown	3,73	2,60E-02
Uncharacterized protein	LOC_Os10g04800	Unknown	3,58	1,12E-03
Uncharacterized protein	LOC_Os08g30014	Unknown	3,51	2,87E-07
Uncharacterized protein	LOC_Os10g40480	Unknown	3,36	4,32E-04
Uncharacterized protein	LOC_Os11g10590	Unknown	3,14	2,78E-06
Uncharacterized protein	LOC_Os07g04980	Unknown	3,10	1,35E-04
Uncharacterized protein	LOC_Os02g06120	Unknown	2,95	8,86E-03
Uncharacterized protein	LOC_Os03g59100	Unknown	2,88	2,67E-02
Uncharacterized protein	LOC_Os02g26794	Unknown	2,53	1,53E-04
Uncharacterized protein	LOC_Os12g37320	Unknown	2,40	1,16E-02
Uncharacterized protein	LOC_Os07g41060	Unknown	2,33	7,19E-05
Uncharacterized protein	LOC_Os10g36180	Unknown	2,33	2,15E-02
Uncharacterized protein	LOC_Os02g39510	Unknown	2,22	4,44E-02
Uncharacterized protein	LOC_Os02g52010	Unknown	2,19	3,22E-02
Uncharacterized protein	LOC_Os07g30240	Unknown	2,18	1,56E-02

Supplemental Table 6. Summary of small RNA sequencing datasets from wild-type and *dcl1a-Ac* plants. Genome sequences were retrieved from MSU Rice Genome Annotation Project release 7. Known miRNAs deposited at miRBase (release 21) are indicated (miRNA families represented by these miRNAs are shown).

	wild-type	***dcl1a-Ac***
Number of reads[(a)]	14 M	18,4 M
Genome matched reads	11 M	15,5 M
t/r/snoRNA matched reads	3.9 M	4.9 M
Unique sRNAs	2 M	2 M
Known miRNAs (families)	455 (256)	452 (257)

(a) "Number of reads" correspond to the sum of reads in the two sequenced libraries from each one biological replicate.

Supplemental Table 7. List of differentially expressed *MIR* genes in *dcl1a-Ac* plants.

miRNA	**Reads[a] *dcl1a-Ac***	**Reads [a] WT(Az)**	**Identity[b]**	**Fold Change[c]**
miR156cg-3p	507,263371	204,019628	0,99	1,31
miR156fhl-3p	552,659984	230,012342	0,99	1,26
miR159a.1	3923,83088	11677,4459	1,00	-1,57
miR159a.2	206,977554	547,931842	0,98	-1,40
miR159d	373,269262	634,105424	0,97	-0,76
miR159f	886,234513	1677,99609	0,96	-0,92
miR162a	1789,19554	2966,89673	1,00	-0,73
miR162b	1742,35682	2894,47728	1,00	-0,73
miR166abcdfj-3p	404143,151	266886,103	1,00	0,60
miR166e-3p	345714,774	229260,962	1,00	0,59
miR166gh-3p	356961,562	237296,162	1,00	0,59
miR166j-5p	919,351653	414,340013	0,99	1,15
miR166m	397138,907	262335,067	1,00	0,60
miR167abc-5p	3181,51405	2094,94418	1,00	0,60
miR168a-3p	16856,8151	30325,6543	1,00	-0,85
miR168a-5p	12165,6061	36758,4671	1,00	-1,60
miR168b	5,4969466	23,9390587	0,97	-2,12
miR169a	499,119149	1015,79928	0,97	-1,03
miR169f	36,5542499	12,7864597	0,98	1,52
miR169no	90,4374919	263,363542	0,96	-1,54
miR169q	4502,99682	1141,58385	1,00	1,98
miR171i-3p	223,11003	482,018734	0,97	-1,11
miR319ab-3p	147,415311	271,86357	0,96	-0,88
miR393a	**364,399103**	**1180,228**	**1,00**	**-1,70**
miR393b-3p	419,478109	1143,01769	1,00	-1,45
miR393b-5p	481,782569	1260,90584	1,00	-1,39
miR396ab-3p	137,548338	23,0917874	1,00	2,57
miR396ab-5p	**3320,31472**	**5313,62374**	**0,95**	**-0,68**
miR396c-3p	210,682152	50,8115495	0,99	2,05
miR396c-5p	**3080,90296**	**5387,19604**	**0,96**	**-0,81**
miR396d	81575,8915	55226,8259	1,00	0,56
miR396ef-5p	85955,141	57270,9981	1,00	0,59
miR396gh	81575,8915	55226,8259	1,00	0,56
miR397a	1278,6518	6669,32412	1,00	-2,38
miR397b	1289,63195	6756,41189	1,00	-2,39
miR398b	**306,218151**	**613,775665**	**0,98**	**-1,00**
miR435	131,636567	20,4554604	0,99	2,69
miR444a-3p	473,977163	881,77564	0,99	-0,90
miR444d	247,939438	406,204364	0,96	-0,71
miR444e	474,351821	886,221997	0,99	-0,90
miR529a	1135,68673	4861,38705	1,00	-2,10
miR529b	**1102,58508**	**4838,88293**	**1,00**	**-2,13**
miR810b	115,590491	308,544527	0,97	-1,42
miR1425-5p	3614,62138	6712,76312	0,99	-0,89
miR1427	180,440641	32,8701177	0,99	2,46
miR1431	**68,3020213**	**17,8029559**	**0,99**	**1,94**
miR1432-5p	627,551314	1148,03839	0,96	-0,87
miR1437b-3p	338,174879	177,492842	0,98	0,93

miR1441	373,592984	200,667303	0,99	0,90
miR1846ab-3p	61,3376178	21,38092	0,96	1,52
miR1846d-5p	58,9270925	28,1349173	0,95	1,07
miR1847	109,42499	22,1758156	1,00	2,30
miR1849	265,727531	125,066378	0,99	1,09
miR1850	457,675225	30,0841001	0,99	3,93
miR1856	932,810938	102,472905	1,00	3,19
miR1859	6,54991127	40,5929828	0,96	-2,63
miR1861d	16,7183859	6,68949996	0,98	1,32
miR1865-3p	30,7888159	128,987706	0,99	-2,07
miR1870-3p	61,0933699	182,06434	0,99	-1,58
miR1876	746,261923	1580,12064	0,99	-1,08
miR1883ab	632,272092	1237,57993	1,00	-0,97
miR2096-3p	17,385999	3,35222901	0,96	2,37
miR2121ab	529,219045	340,974531	0,97	0,63
miR2865	104,097936	38,8696247	0,98	1,42
miR2867-3p	20,6987656	5,46219101	0,98	1,92
miR2869	27,7729191	10,6556443	0,97	1,38
miR2870	33,2217405	7,93511986	1,00	2,07
miR2871a-5p	776,295856	1388,71706	0,97	-0,84
miR2874	107,2098	18,6582962	0,99	2,52
miR2877	401,620828	1357,03442	0,99	-1,76
miR2878-3p	209,172398	400,300416	0,97	-0,94
miR2878-5p	265,485723	459,149731	0,97	-0,79
miR3982-3p	173,044171	44,3475523	0,99	1,96
miR5072	86,96667	280,375744	0,96	-1,69
miR5146	10,6623123	28,1786252	0,97	-1,40
miR5148abc	199,783103	83,5033621	0,99	1,26
miR5508	36,3478354	11,9440768	0,99	1,61
miR5513	79,4222597	23,6375181	0,98	1,75
miR5804	8,29814376	2,75313547	0,98	1,59
miR5809	214,135981	61,6543027	0,99	1,80
miR5813	13,4211833	4,56545714	0,97	1,56
miR5827	836,117344	431,570382	0,96	0,95
miR5829	1,84727101	4,34815688	0,96	-1,24
miR6246	42,636708	6,68776911	0,97	2,67
miR6248	113,665245	26,5849002	0,99	2,10
miR6249ab	132,818219	62,3939461	0,98	1,09
miR6253	1,8591125	5,1138915	0,96	-1,46
miR7694-5p	262,012156	522,506193	0,97	-1,00
miR7695-3p	1707,60207	558,837676	0,99	1,61
miR7695-5p	924,903018	375,723021	0,98	1,30

Marked in bold are the validated miRNAs

(a) miRNA reads expressed as Reads Per Kilobase Million (RPKM)

(b) Probability of being a true-positive differentially expressed gene

(c) Fold change expressed as Log_2

Chapter III

The microRNA miR773 is involved in the Arabidopsis immune response to fungal pathogens

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Abstract

MicroRNAs (miRNAs) are 21-24 nucleotide short non-coding RNAs that trigger gene silencing in eukaryotes. In plants, miRNAs play a crucial role in a wide range of developmental processes and adaptive responses to abiotic and biotic stresses. In this work, we investigated the role of miR773 in modulating resistance to infection by fungal pathogens in *Arabidopsis thaliana.* Interference with miR773 activity by target mimics (in *MIM773* plants) and concomitant up-regulation of the miR773 target gene *METHYLTRANSFERASE 2* (*MET2*) increased resistance to infection by necrotrophic (*Plectosphaerrella cucumerina*) and hemibiotrophic (*Fusarium oxysporum*, *Colletotrichum higginianum*) fungal pathogens. By contrast, both *MIR773* overexpression and *MET2* silencing enhanced susceptibility to pathogen infection. Upon pathogen challenge, *MIM773* plants accumulated higher levels of callose and reactive oxygen species than wild-type plants. Stronger induction of defense gene expression was also observed in *MIM773* plants in response to fungal infection. Expression analysis revealed an important reduction in miR773 accumulation in rosette leaves of plants upon elicitor perception and pathogen infection. Taken together, our results not only show that miR773 mediates PAMP (pathogen-associated molecular pattern)-triggered immunity (PTI), but also demonstrate that suppression of miR773 activity is an effective approach to improve disease resistance in Arabidopsis plants.

Introduction

Plants have evolved diverse mechanisms to defend themselves against pathogenic microorganisms. The initiation of defense responses typically begins with the recognition of conserved pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRR), which leads to PAMP-triggered immunity (PTI) (Jones and Dangl 2006; Boller and He 2009). During PTI, plants express a diverse array of immune responses, such as callose deposition and generation of reactive oxygen species (ROS), activation of Ca^{2+} signalling and protein phosphorylation cascades which ultimately leads to the dynamic reprogramming of host gene expression and accumulation of Pathogenesis-Related (PR) proteins (Van Loon et al. 2006; Bigeard et al. 2015). Among the PAMPs triggering defenses against pathogens are the bacterial (flagellin) and fungal (chitosan) elicitors (Zipfel et al. 2004; Luna et al. 2011). However, in order to survive successfully, pathogens have to produce and deliver effectors that effectively suppress PTI, resulting in disease susceptibility. As a counteractive strategy, plants have evolved a repertoire of immune receptors, or resistance (R) proteins, that recognize pathogen effectors to mount an immune response, the so called effector-triggered immunity (ETI) (Jones and Dangl 2006; Cui et al. 2015). The plant hormones ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) play central roles in the regulation of plant immune responses (Glazebrook 2005; Robert-Seilaniantz et al. 2011; Denancé et al. 2013; Vleesschauwer et al. 2014). While many of these responses rely on gene expression changes due to transcriptional activation or repression, there is increasing evidence for post-transcriptional regulation of immune responses, with host endogenous small RNAs being essential in both PTI and ETI (Katiyar-Agarwal and Jin 2010; Staiger et al. 2012; Pumplin and Voinnet 2013; Seo et al. 2013; Huang et al. 2016).

MicroRNAs (miRNAs) are a class of short non-coding small RNAs that direct post-transcriptional gene silencing in a sequence-specific manner through cleavage or translational repression mechanisms (Llave et al. 2002; Brodersen et al. 2008, Axtell 2013; Rogers and Chen 2013). miRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in Arabidopsis (Li et al. 2013a). MiRNAs play a critical role in controlling developmental processes (Rubio-Somoza and Weigel 2011; Li and Zhang 2016) and adaptation to different types of abiotic (e.g. nutrient deficiency, drought, cold and salinity) and biotic stresses (Chiou et al. 2006; Navarro et al. 2006; Jagadeeswaran et al. 2009;

Shivaprasad et al. 2012b; Sunkar et al. 2012; Campo et al. 2013b; Li et al. 2014b; Huang et al. 2016c). Our knowledge of miRNA functions in plant immunity is, however, still limited.

The role of miRNAs in disease resistance was first described in the interaction of *Arabidopsis thaliana* (Arabidopsis) with the bacterial pathogen *Pseudomonas syringae* (Navarro et al. 2006). Here, perception of flg22, a flagellin-derived peptide, promotes miR393 accumulation resulting in down-regulation of auxin receptors and in bacterial resistance. Subsequently, several other miRNAs have been shown to play a role during defense in Arabidopsis, such as miR393, miR396, miR398b, miR40l0, miR472, miR844 and miR863 (Navarro et al. 2006; Li et al. 2010c; Boccara et al. 2014; Park et al. 2014; Lee et al. 2015; Niu et al. 2016; Soto-Suarez et al. 2017). miRNAs might function as positive or negative regulators in PTI and ETI by targeting negative or positive defense regulators. Although an important fraction of the Arabidopsis miRNAome is known to be regulated during pathogen infection (Jagadeeswaran et al. 2009; Baldrich et al. 2014), the function of most of these pathogen-regulated miRNAs, and their contribution to Arabidopsis immunity, remains elusive. Additionally, most studies so far carried out for the identification of miRNAs affecting disease resistance defense have been executed in the interaction of Arabidopsis with the bacterial pathogen *Pseudomonas syringae*, and our knowledge on miRNAs controlling immune responses to infection by fungal pathogens in Arabidopsis is still scarce.

In the present work, we examined the role of miR773 in Arabidopsis immunity to fungal infection. We show that blocking miR773 activity through the expression of a miR773 target mimic in *MIM773* lines and the concomitant up-regulation of the miR773 target gene *MET2* (Fahlgren et al. 2007; Li et al. 2010) confer resistance to infection by necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Fusarium oxysporum* f. sp. *conglutinans*, *Colletotrichum higginsianum*) fungal pathogens. Conversely, overexpression of *MIR773A* and silencing of *MET2* increased susceptibility to fungal infection. Resistance to infection by *P. cucumerina* in *MIM773* lines is associated to a stronger expression of basal defense responses upon pathogen attack, including higher accumulation of H_2O_2 and callose, and superactivation of defense gene expression. Collectively, our results support a role of miR773 in controlling disease resistance in Arabidopsis.

Material and Methods

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Col-0 plants were grown on a mixture of soil, perlite and vermiculite (2:1:1) under neutral day conditions (12h light/12h dark), at 22°C/20°C day/night, and 60% relative humidity. The lines used in this study are listed in Supplemental Table 1. The production of *MIM* lines (Col-0 background) was previously described (Todesco et al. 2010). Rosette diameter and leaf number were determined in 3 weeks-old plants using Image J. The Arabidopsis mutants used in this work, *met2-1* and *met2-2* (SALK_010893 and SALK_010896, respectively) were grown as described above. Primers used for genotyping the T-DNA insertion mutants are indicated in Supplemental Table 2.

For *MIR773A* overexpression, a 364-bp genomic sequence containing the miR773a precursor was obtained by PCR from Arabidopsis genomic DNA using gene-specific primers (Supplemental Table 2). The miR773a precursor sequence was cloned into the pCAMBIA1300 vector under the control of the *35SCaMV* promoter. The floral dip method was used for Arabidopsis (Col-0) transformation. As a control, Arabidopsis plants were transformed with the pCAMBIA1300 empty vector.

Infection assays and elicitor treatment

Fungi were grown at 28ºC on PDA (potato dextrose agar) supplemented with chloramphenicol. Spores were collected by adding sterile water to the surface of the mycelium and adjusted to the appropriate concentration using a Bürker counting chamber. For infection experiments, control (wild-type, empty vector) and *MIM* plants (homozygous lines) were grown for 3 weeks and then spray-inoculated with a spore suspension of *P. cucumerina* at the appropriate concentration, or mock-inoculated. Progression of the infection was examined at different days post inoculation (dpi). I*n the case of F. oxysporum f. sp. conglutinans,* the spore suspension was applied to the soil near the roots (200µl; $1x10^6$ spores/mL). Infections with *C. higginsianum* were performed by drop inoculation of the leaves (10µl, 5×10^5 spores/mL). Fungal-inoculated and mock-inoculated plants were allowed to continue growth under high humidity. Statistically significant differences among the inoculated Arabidopsis genotypes were determined by one-way ANOVA. Disease severity and lesion area were determined using digital imaging software (Assess 2.0, American

Phytopathological Society). The relative quantification of fungal DNA in infected leaves was carried out by qPCR as described (Sánchez-Vallet et al. 2010; Soto-Suárez et al. 2017). Primers are listed in Supplemental Table 2. For elicitor treatment, Arabidopsis plants were sprayed with an elicitor suspension obtained from *P. cucumerina* (300 µg/mL) as previously described (Coca and San Segundo 2010).

Plant tissue staining

Trypan blue staining was performed as previously described (Ramírez et al. 2010). Stained material is observed using a Zeiss Axiophot microscope under bright-field illumination. At least 15 plants per genotype were analyzed.

Callose deposition was determined by aniline blue staining. For this, chlorophyll was removed from leaves with 70% ethanol which were then incubated in 70 mM phosphate buffer (pH 9.0) supplemented with aniline blue (0.01% w/v) with vacuum for 30 minutes. Samples were maintained in dark conditions for 2 hours. Leaves were observed with an epifluorescence microscope (Zeiss Axiophot microscope) under UV illumination. Callose deposition was quantified by determining the relative number of callose-corresponding pixels relative to the total number of pixels covering the plant material on digital photographs (Luna et al. 2011).

DAB (3,3′-diaminobenzidine) staining was used to determine H_2O_2 levels. For this, Arabidopsis plants were immersed in a DAB solution (1 mg/mL) with vacuum for 30 min, maintained in the dark overnight, and washed with 70% ethanol for 1 h. Plants were observed using an Olympus DP71 microscope under bright-field illumination. DAB staining intensities were quantified from digital photographs by the number of dark-brown DAB pixels relative to the leaf surface.

Callose measurements and H_2O_2 quantifications were made on at least 15 different seedlings per genotype. Statistically significant differences were determined by one-way ANOVA test.

Gene expression analyses

Total RNA was extracted using TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from TURBO^{TM} DNAse (Ambion) treated total RNA (1 µg) with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and oligo-dT. RT-qPCRs were performed in optical 96-well plates in a Light Cycler 480 using SYBR® Green (Roche). Primers were designed using Oligo Analyzer software (Integrated DNA technologies, Coralville, Iowa, USA). The *β-tubulin2* (At5g05620) expression was used to normalize the transcript level in each sample. Three independent biological replicates with three technical replicates were analyzed. ANOVA test was used to evaluate differences in gene expression. Accumulation of mature miR773 sequences was determined by stem-loop reverse transcription quantitative PCR (SL-RT-qPCR) (Varkonyi-Gasic et al. 2007) using primers listed in **Supplemental Table 2**. Nucleotide sequencing confirmed the specific amplification of miR773 sequences. For Northern blot analysis, small RNA fraction was obtained from 600 µg of total RNAs, fractioned in a 17’5% denaturing polyacrylamide gel containing 8M urea and transferred to nylon membranes. Probes were 3’-labeled with digoxigenin using the DIG oligonucleotide 3’-End Labeling kit following manufacturer’s instructions (Roche).

MiR773 *in situ* hybridization

Plants grown at *in vitro* conditions during 15 days were inoculated with *P. cucumerina* spores, or mock-inoculated. Mature leaves were harvested at 24 hpi and analyzed separately for *in situ* miR773 detection. Fixation and sample processing was performed as described in Wang et al. 2008. Hybridization was carried out with a 3’digoxygenin-labeled locked nucleic acid (LNA)-miR773a probe (Exiqon) overnight at 37ºC. Detection of the hybridized probe was performed using an antibody solution (anti-DIG, alkaline phosphatase conjugated antibody) and the color substrate NBT (4-nitroblue-tetrazolium chloride).

Results

Resistance to infection by fungal pathogens in *MIM773* plants

To identify miRNAs contributing to disease resistance in *A. thaliana*, we initially assayed resistance to infection by *P. cucumerina* in a set of miRNA target mimicry (*MIM*) lines in which the activity of miRNAs is suppressed and the activity of miRNA targets correspondingly increased (Todesco et al. 2010). *P. cucumerina* (previously known as *Fusarium tabacinum*; anamorph *Plectosporium tabacinum*) (Palm et al. 1995) is a necrotrophic fungus that causes sudden death and blight in crops such as melon, soybean, snap bean, pumpkin, squash, zucchini, or white lupine, and that can also infect Arabidopsis (Ton and Mauch- Mani 2004; Llorente et al. 2005; Sánchez-Vallet et al. 2012).

Among the various *MIM* lines assayed (**Supplementary Table 1**), *MIM773* plants were consistently more resistant to infection by *P. cucumerina* than wild-type and empty vector controls (**Fig. 1A**). Disease severity was defined as the number of plants falling into disease categories distinguished on the basis of the number of diseased leaves. At 10 days after infection (dpi), the *MIM773* plants had a disease score of 0 (plants with no symptoms) and 1 (plants with up to 25% of diseased leaves). By the same time, control plants scored in categories 2 (26%-50% of diseased leaves) and 3 (51%-100%) (**Fig. 1A**). Measurements of fungal biomass by quantitative PCR (qPCR) (**Fig. 1A**) and trypan blue staining (**Fig. 1B**) confirmed reduced fungal growth in leaves of *P. cucumerina*-inoculated *MIM773* plants compared to *P. cucumerina*-inoculated control plants.

To ascertain that the observed effects were due to changes in miR773 activity, we assayed miR773 accumulation and miR773 target gene expression. In Arabidopsis, there are two *MIR773* family members, with miR773a having been shown to target *MET2* (At4g14140) (Fahlgren et al. 2007; Li et al. 2010). As expected, *MIM773* plants accumulated lower levels of mature miR773 as a consequence of mimicry triggered miRNA degradation (Todesco et al, 2010) (**Fig. 1C; Supplementary Fig. 1**). As a control, miR396 accumulation was found not to be affected in *MIM773* plants relative to control (wild-type, empty vector plants) (**Supplementary Fig. 2**). The observed reduction in mature miR773 levels was paralleled by an increase in *MET2* transcript levels (**Fig. 1D**). *MIR773A* precursor transcripts accumulated at similar levels in *MIM773* and wild-type plants (**Fig. 1E**). Finally, the *MIM773* plants were similar to wild-type plants in terms of growth, and rosette leaf number and diameter (**Supplementary Fig. 3**).

Next, we challenged *MIM773* plants with the hemibiotrophic fungi *Fusarium oxysporum* f. sp. *conglutinans* (*FOC*) and *Colletotrichum higginsianum*, the causal agents of wilt and of anthracnose leaf spot disease on a broad range of cultivated plant species as well as Arabidopsis (Mauch-Mani and Slusarenko 1993; O'Connell et al. 2004). Upon *FOC* inoculation, chlorosis appeared by 5-7 days post-inoculation in leaves of control plants and progressively progressed over time, whereas by *MIM773* lines exhibited much milder symptoms (**Fig. 2A**). Disease severity scores and fungal biomass supported a suppressive effect in *MIM773* plants (**Fig. 2A**, right panels**).** *MIM773* plants also were more resistant to *C. higginsianum* (**Fig. 2B**), with less diseased leaf area and reduced fungal biomass accumulation (**Fig. 2B**, right panels).

Taken together, these results demonstrate that reduced miR773 activity enhances resistance to infection by both necrotrophic and hemibiotrophic fungi.

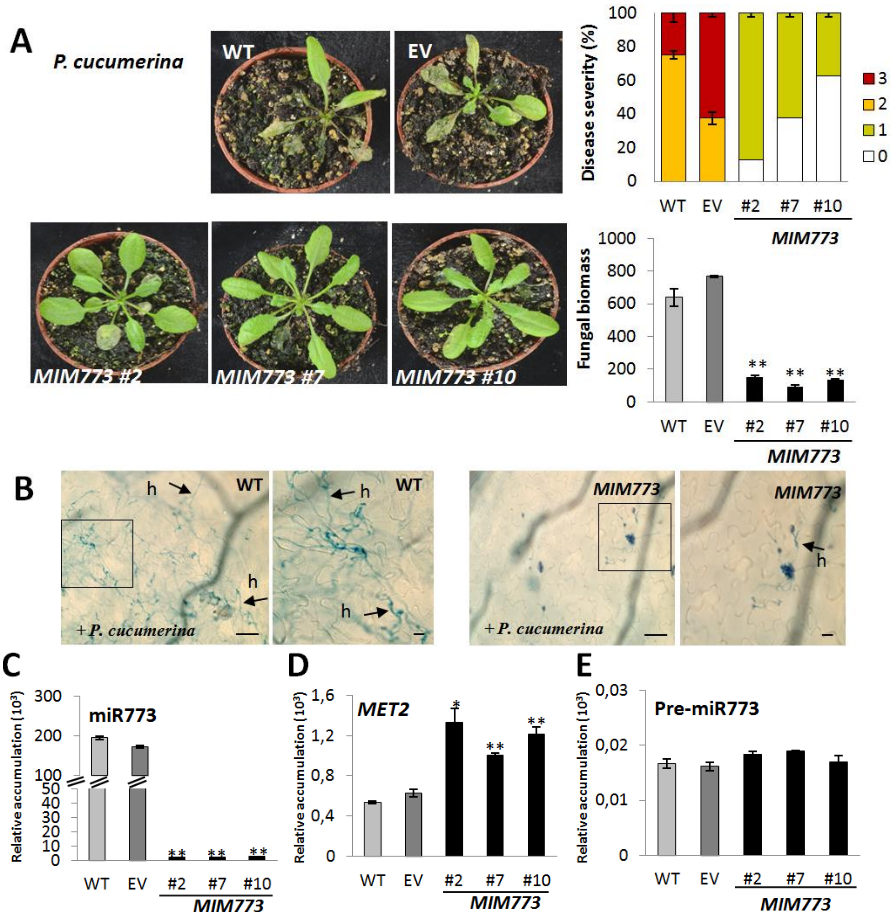

Figure 1. ***MIM773*** **plants show resistance to infection by** ***P. cucumerina*****.** Three-week old plants were inoculated with fungal spores. Results are from one out of three independent experiments performed with three independent *MIM773* lines (*MIM773#2*, *MIM773#7* and *MIM773#10*) and control plants (WT, empty vector) which gave similar results. At least 12 plants/genotype were assayed in each experiment. Histograms show the mean ± SD. Statistical significance (ANOVA test, **, P ≤ 0.01). **A,** Plants 10 days after spray-inoculation with *P. cucumerina* spores ($1x10^6$ spores/mL; 0.5 mL per plant). Disease severity was scored as the number of leaves showing disease symptoms: 0, no symptoms; 1, 1–25%; 2, 26–50%; 3, 51–100%. Quantification of fungal biomass was carried out by qPCR using specific primers of *P. cucumerina β-tubulin* at 3 dpi (Sánchez-Vallet et al. 2010). Values of fungal DNA were normalized to the Arabidopsis *UBIQUITIN21* gene (At5g25760). **B,** Trypan blue staining of *P. cucumerina*-inoculated leaves at 24 hpi. Right panels show the boxed region at higher magnification (scale bar, 200 µm). h, hyphae. **C,** Accumulation of mature miR773 was determined by stem-loop reverse transcription quantitative PCR. **D,** Expression of the miR773 target *MET2*, as determined by RT-qPCR. **E,** Accumulation of miR773a precursor transcripts, as determined by RT-qPCR.

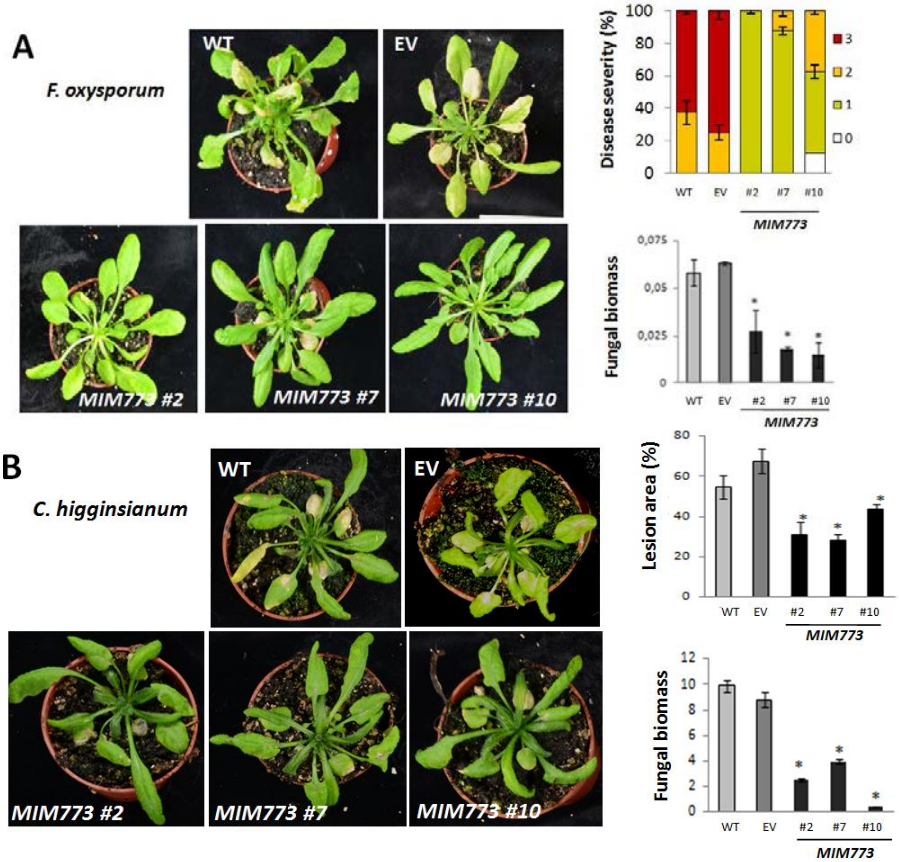

Figure 2. ***MIM773*** **plants show resistance to infection by hemibiotroph fungal pathogens.** Three-week old plants were inoculated with fungal spores. Results are from one out of three independent experiments performed with three independent *MIM773* lines (*MIM773#2*, *MIM773#7* and *MIM773#10*) and control plants (wild-type, empty vector) which gave similar results. At least 12 plants/genotype were assayed in each experiment. Histograms show the mean ± SD. Statistical significance (ANOVA test, *, $P \leq 0.05$). **A,** Plants 17 days after inoculation with *F. oxysporum* f. sp. *conglutinans*. Inoculation was carried out by applying the fungal spores ($1x10^6$ spores/mL) to soil. Disease severity was determined as described above. Quantification of *F. oxysporum* DNA was carried out by qPCR using specific primers of the *F. oxysporum chitinase class III* gene at 3 dpi (Bravo-Ruiz et al. 2016). Values are fungal DNA levels normalized against the Arabidopsis *UBIQUITIN21* gene (At5g25760). **B,** Plants at 9 days after inoculation with *C. higginsianum.* Leaves were locally inoculated with a spore suspension at $5x10^5$ spores/mL. Diseased leaf area was quantified using image analysis software (Assess 2.0). Quantification of *C. higginsianum* DNA was carried out by qPCR using specific primers for the *C. higginsianum* ITS2 (*Internally transcribed spacer 2*) gene at 3 dpi. Values are fungal DNA levels normalized against the Arabidopsis *UBIQUITIN21* gene.

MIM773 plants exhibit stronger PTI upon *P. cucumerina* infection

A common feature of basal resistance to pathogen infection is the accumulation of callose and ROS production at the infection sites (Torres et al. 2006; Luna et al. 2011; Pastor et al. 2013; Morales et al. 2016; Pétriacq et al. 2016). Callose functions as the first-line of defense impeding fungal penetration, whereas ROS act as antimicrobial agents and signalling molecules for the activation of defense responses. Aniline blue staining indicated that *MIM773* plants inoculated with *P. cucumerina* accumulated more callose than wild-type plants and produced more H_2O_2 (**Fig. 3A and B).** It is noteworthy that in *MIM773* plants callose and H_2O_2 were not detected before inoculation with *P. cucumerina*, indicating that these immune responses were not constitutively active in *MIM773* plants. The enhanced callose and H_2O_2 accumulation in leaves of *MIM773* plants might well help to arrest pathogen growth in these plants.

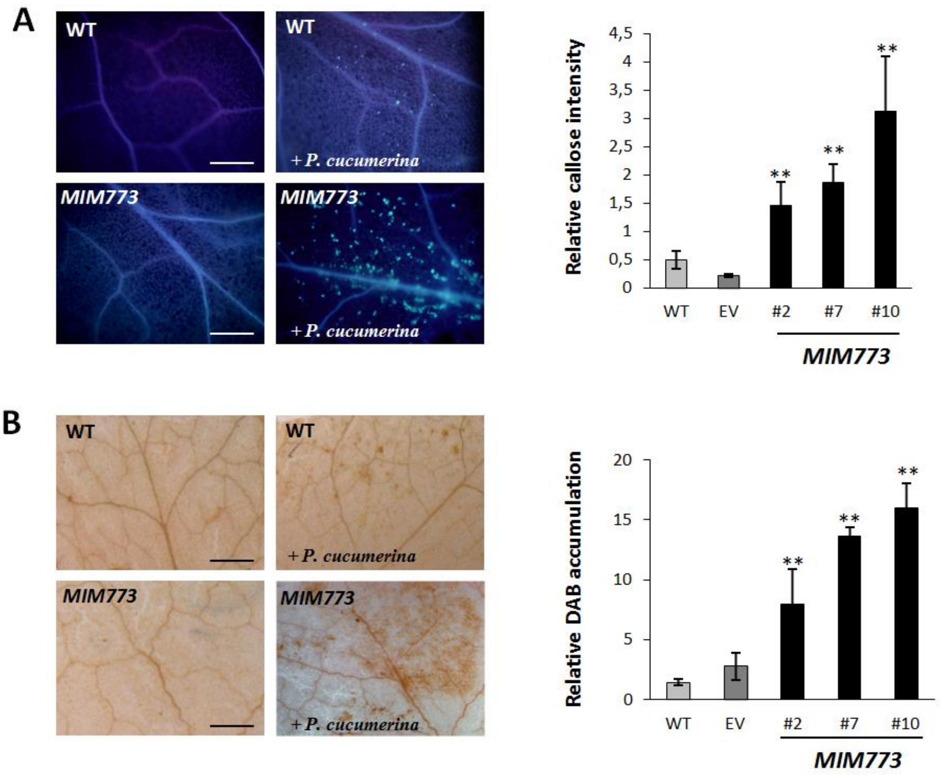

Figure 3. Callose deposition and H_2O_2 production in *P. cucumerina*-infected leaves of wild-type and *MIM773* plants. *In vitro* grown plants were sprayed with a *P. cucumerina* spore suspension ($1x10^6$ spores/mL, 2 mL per plate). Statistical significance was determined by ANOVA (**, $P \leq 0.01$). Representative results of one of three independent experiments that produced similar results are presented (3 independent lines and 15 plants per independent line were assayed, and 4 leaves per plant were examined). Histograms (right panels) represent mean ± SD. **A,** Callose deposition in mock-inoculated (left panels) and *P.cucumerina*-inoculated (right panels) plants was examined by aniline blue staining at 24 hpi. Callose deposition was quantified by determining the number of callose-corresponding pixels relative to the total number of pixels covering plant material on digital photographs. Bars (left panels) represent 50 µm. **B,** H_2O_2 production was visualized by DAB staining at 24 hpi. Patches of brown precipitate of oxidized DAB are prominent in *MIM773* plants. Bars (left panels) represent 50 µm.

MIR773A overexpression and *MET2* silencing confers enhanced susceptibility to *P. cucumerina*

Since *MIM773* plants exhibited enhanced resistance to fungal infection, we reasoned that either constitutive expression of *MIR773A* or silencing of *MET2* expression might result in susceptibility to pathogen infection. We generated plants overexpressing *MIR773A* (*MIR773A* OE), which accumulated higher levels of mature miR773 and had reduced levels of *MET2* transcripts (**Fig. 4A**). Northern blot analysis confirmed miR773 accumulation in *MIR773 OE* plants (**Supplementary Fig. 1**). In the absence of infection, *MIR773A OE* plants showed no obvious differences in growth, leaf number and rosette diameter to non-transgenic plants (**Supplementary Fig. 4**).

Three-week old *MIR773A* OE and control plants were challenged with *P. cucumerina*. *MIR773A* overexpression increased susceptibility to *P. cucumerina* (**Fig. 4B**). Disease susceptibility in *MIR773A* OE plants was further confirmed by quantifying disease severity and fungal biomass in the infected plants (**Fig. 4C**). The opposite effects of depleting miR773 in *MIM773* plants and overexpression of miR773 in *MIR773A* OE plants are in agreement with miR773 negatively regulating resistance to *P. cucumerina*.

We confirmed that in two independent T-DNA insertion lines for *MET2* (*met2-1*, SALK_010893 and *met2-2,* SALK_010896) the *MET2* locus is disrupted by insertions in the second exon (**Supplemental Fig. 5A**). *MET2* transcripts were not detected in the *met2* mutants supporting that they are knock-out mutants for *MET2* (**Supplemental Fig. 5B**, left panel). As expected, *MIR773A* precursor expression was unaffected in the *met2* mutants (**Supplemental Fig. 3B**, right panel), which showed no obvious morphological defects in the absence of infection (**Supplemental Fig. 5C**).

Upon challenge with *P. cucumerina*, both *met2* mutant lines exhibited enhanced susceptibility to infection, supported by visual inspection, disease severity scoring and quantification of fungal biomass in the infected plants (**Fig. 4B and C**). This finding is consistent with the observed phenotype of susceptibility to *P. cucumerina* in *MIR773A* OE plants, and provides additional support for the involvement of the miR773/*MET2* pair in disease resistance.

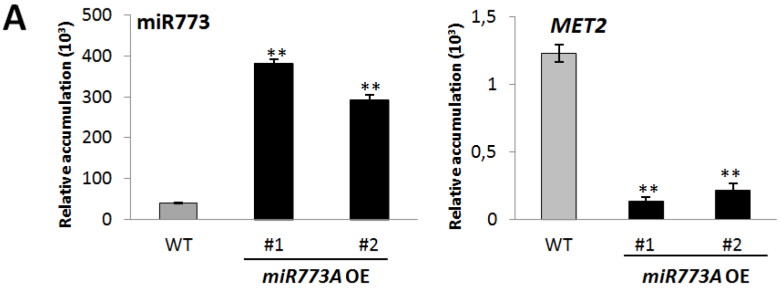

Figure 4. Enhanced susceptibility to infection by *P. cucumerina* in transgenic plants overexpressing *MIR773A* and *met2* loss-of-function mutants. Three independent homozygous *MIR773A* OE plants, *met2* and control plants (EV, empty vector; WT, wild-type) were analyzed. Three independent experiments were carried out each with at least 12 plants per line. Results for two *MIR773A* OE lines are shown (similar results were obtained for the third *MIR773A* OE line). **A**, Accumulation of miR773 (left panel) and *MET2* transcripts (right panel) in OE miR773a plants. The histogram shows the mean ±SD (ANOVA test; ** P ≤ 0.01). **B**, Disease susceptibility of OE miR773a and *met2* plants at 15 dpi with *P. cucumerina* spores (1x10^4 spores/mL). **C**, Disease severity and fungal biomass were determined as indicated in Fig. 1. Statistical significance was determined by ANOVA (**, P ≤ 0.01, overexpressor or mutant lines *vs* wild-type).

miR773 accumulation decreases as part of PTI responses against fungal pathogens

Because altering miR773 activity has an effect on the host response to pathogen infection, but does not appear to have consequences in the absence of pathogens, we sought to determine whether miR773 accumulation was itself pathogen responsive. As shown in **Figure 5A**, steady-state-levels of *MIR773* transcripts and mature miR773 rapidly decreased after infection with *P. cucumerina*, while expression of the miR773 target *MET2* was increased (**Fig. 5B**).

In PTI, the induction of defense mechanisms relies on the detection of PAMPs (or elicitors). We therefore investigated whether miR773 might be induced by a crude preparation of fungal elicitors. Similar to *P. cucumerina* infection, elicitor treatment rapidly reduced both miR773 precursor and mature miRNA (**Fig. 5C**), and at the same time enhanced *MET2* expression (**Fig. 5D**). These observations indicate that miR773 is PAMP-responsive and functions in PTI.

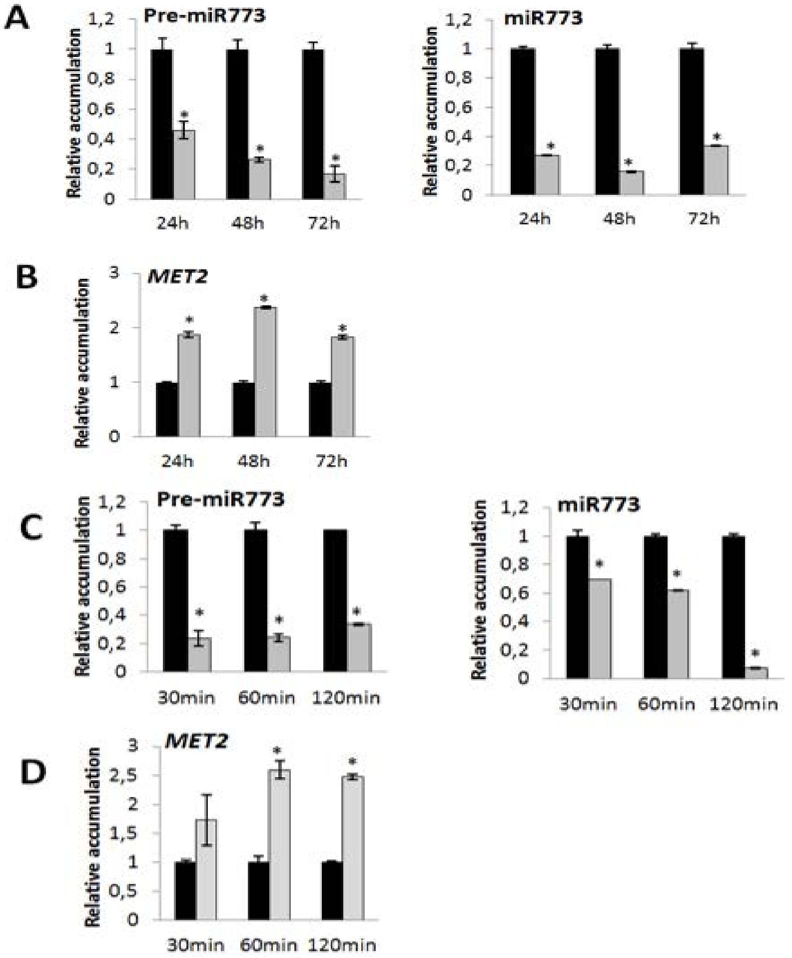

Figure 5. Accumulation of miR773 and *MET2* transcripts in rosette leaves in response to infection by *P. cucumerina* or treatment with elicitors obtained from *P. cucumerina*. The values represent changes in the accumulation of miR773 sequences at the indicated times after inoculation with fungal spores (A, B), treatment with fungal elicitors (C, D), or mock-inoculation. Black and grey bars correspond to mock-inoculated and *P. cucumerina*-inoculated plants, respectively. The expression level in mock-inoculated plants was set to 1.0. Histograms show the mean ± SD of one out of 3 biological replicates with similar results, each replicate with 12 plants per genotype (*, P ≤ 0.05; ANOVA test). **A**, Accumulation of pre-miR773 and mature miR773 sequences after inoculation with *P. cucumerina* spores as determined by RT-qPCR and stem-loop RT-qPCR analysis, respectively. **B,** Expression of *MET2* in response to inoculation with *P. cucumerina* spores. **C,** Accumulation of pre-miR773 and mature miR773 sequences at the indicated times of treatment with *P. cucumerina* elicitors. **D,** Expression of *MET2* in response to treatment with *P. cucumerina* elicitors.

For a better understanding of miR773 regulation, we investigated miR773 accumulation in root and leaf tissues of wild-type plants in response to fungal infection. In unchallenged plants, miR773 expression was much higher in leaves than in roots (**Fig. 6**). Upon infection with either *P. cucumerina* or *C. higginsianum* significantly decreased miR773 accumulation in leaves, but not in roots, of wild-type plants (**Fig. 6A**, left and middle panels). In contrast, infection with the root pathogen *F. oxysporum* f. sp. *conglutinans* causes a reduction in miR773 levels in both tissues (leaves and roots) (**Fig. 6A**, right panel). *In situ* hybridization revealed miR773 accumulation in leaf mesophyll cells of non-infected plants (**Fig. 6B**, upper left panel), while miR773 was barely detectable in leaves of either uninfected and *P. cucumerina*-infected *MIM773* plants, or infected wild-type plants (**Fig. 6B**, upper panels). Collectively, these results demonstrate that miR773 accumulation in mesophyll cells of rosette leaves decreases in response to infection by *P. cucumerina*.

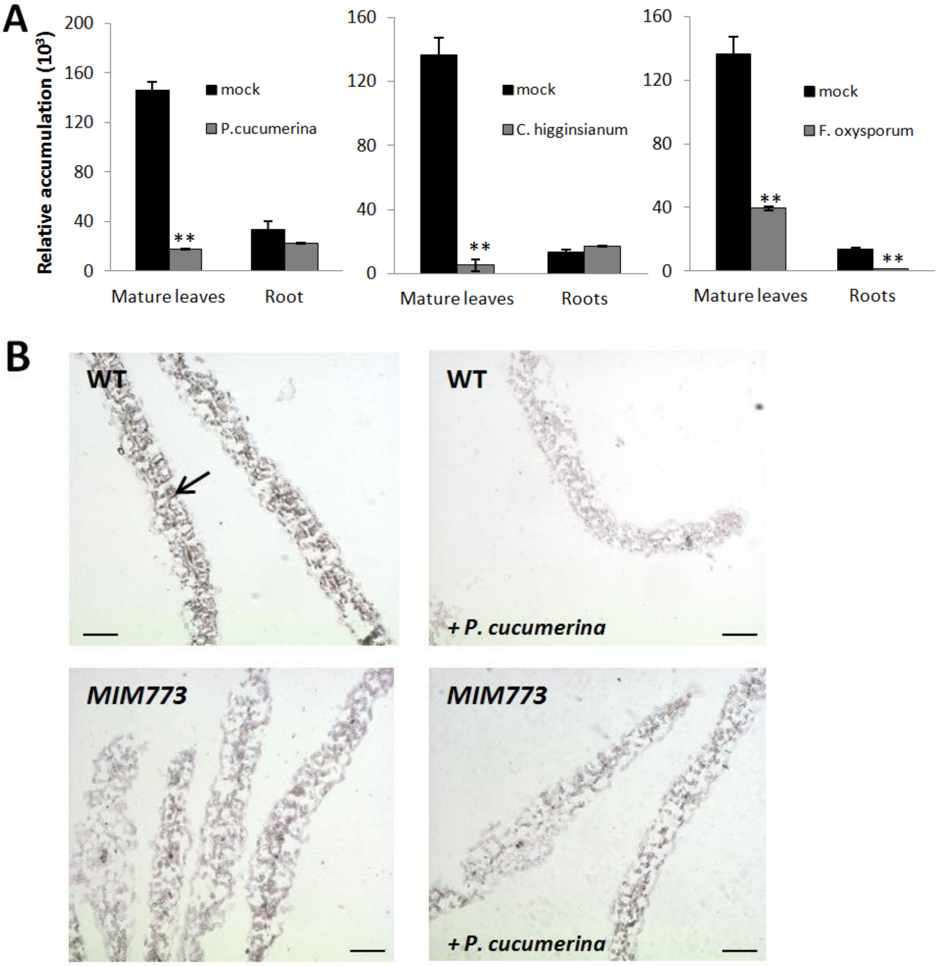

Figure 6. Detection of miR773 in wild-type and *MIM773* plants. A, Accumulation of miR773 was determined by stem-loop RT-PCR analysis in leaves and roots of *in vitro*-grown Arabidopsis plants at 24 hpi with fungal spores (*P. cucumerina,* 1x10^6 spores/mL, *C. higginsianum,* 5x10^5 spores/mL, *F. oxysporum* f. sp. *conglutinans,* 1x10^6 spores/mL) (grey bars). Control plants were mock-inoculated (black bars). Histograms represent the mean ± SD of one out of 3 biological replicates with similar results, each replicate with 24 plants per genotype. Statistical significance was determined by ANOVA (** P $\leq$ 0.01, infected *vs* mock-inoculated). **B,** *In situ* miR773 hybridization in mature leaves of *P. cucumerina*-inoculated and mock-inoculated plants. Hybridization was carried out using a 3'digoxygenin-labeled LNA miR773a probe (scale bar, 500 µm).

miR773 reduction leads to stronger induction of defense gene expression

To ascertain the molecular basis of the enhanced resistance to *P. cucumerina* in *MIM773* plants, we studied the expression of defense marker genes associated with JA/ET (*PDF1.2*), JA (*VSP2*) and SA (*PR1*, *NPR1*) signalling. Defense gene expression was induced in response to fungal infection in wild-type plants (**Fig. 7A and B**). Most importantly, transcripts of all defense genes accumulated to higher levels in *P. cucumerina*-inoculated *MIM773* plants than in *P. cucumerina*-inoculated wild-type plants (**Fig. 7A and B**). Contrary to this, a lower induction (*VSP2*, *PR1a*), or no induction (*PDF1.2*) of defense gene expression was observed in fungal-infected *met2* plants compared to the fungal-infected wild-type plants (**Supplemental Fig. 6**). These observations support that, upon challenge with *P. cucumerina*, the *MIM773* plants show stronger activation of defense responses that are regulated by ET, JA or SA, consistent with the enhanced disease resistance in these plants. In addition, under non infection conditions, the expression of *PDF1.2* was slightly higher in *MIM773* plants (**Fig. 7A**).

When examining the expression of genes involved in ET biosynthesis in *MIM773* plants, namely *ACS1* (*1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 1*) and *ACO2* (*ACC OXIDASE 2*), they were found to be induced more strongly in *MIM773* plants than in wild-type plants in response to *P. cucumerina* infection (**Fig. 7C**). In the absence of pathogen infection, *ACS1* and *ACO2* expression was already slightly elevated in *MIM773* plants (**Fig. 7C**).

These pieces of evidence support that *MIM773* plants respond more strongly to pathogen infection through the superinduction of defense genes whose expression is regulated by defense-related hormones. An opposite response occurs in *met2* plants, these plants exhibiting a weaker induction (even no induction) of defense gene expression in response to fungal infection.

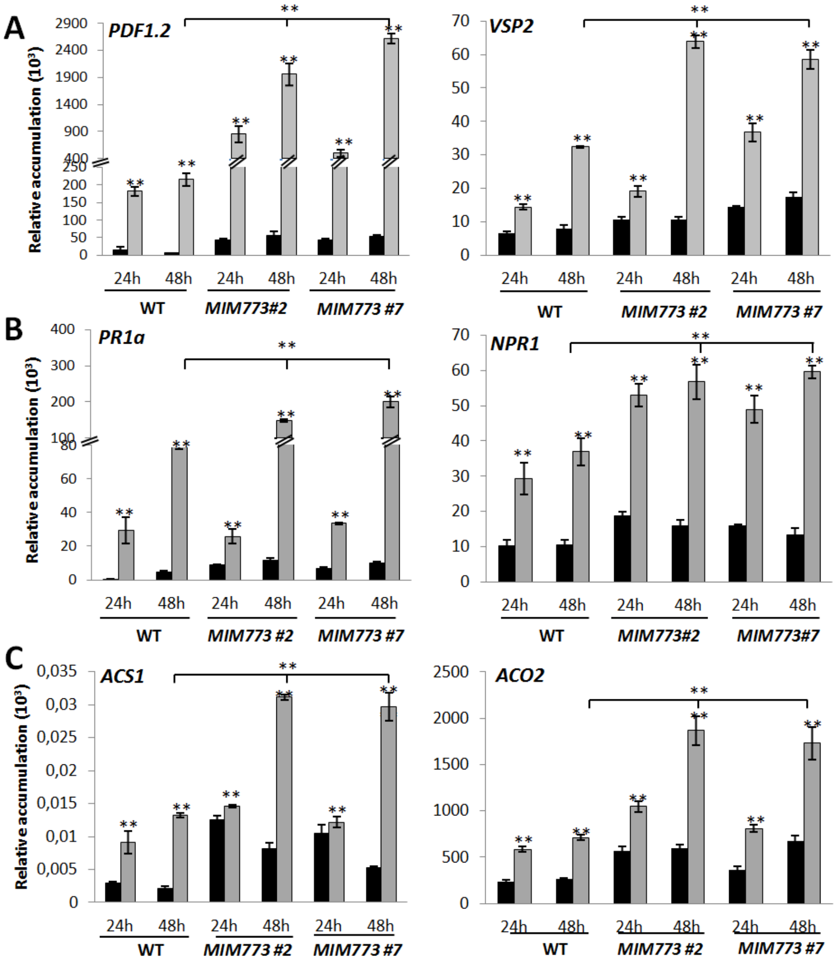

Figure 7. Accumuation of defense marker genes in wild-type and *MIM773* plants. RT-qPCR analysis showing the accumulation of transcripts for genes that are regulated by the JA/ET-dependent (*PDF1.2*), JA-dependent (*VSP2*) and SA-dependent (*PR1a*, *NPR1*) signalling pathways at the indicated time points after inoculation with *P. cucumerina* spores. Analysis of the *ACS1* and *ACO2* genes involved in ET biosynthesis is shown. Mock-inoculated and *P.cucumerina*-inoculated plants are represented by black and grey bars, respectively. Results from 2 out of the 3 *MIM773* lines analyzed are shown (similar results were obtained for the third line). Histograms show the mean ± SD of 3 biological replicates, each with 12 plants per genotype. Statistical significance was determined by ANOVA (**, P ≤ 0.01, *P. cucumerina*-inoculated *vs* mock-inoculated; wild-type *P. cucumerina* –inoculated 48h *vs MIM773 P.cucumerina* –inoculated 48h).

Discussion

In this study, we provide evidence that miR773a functions as a negative regulator of Arabidopsis immunity against necrotrophic (*P. cucumerina*) and hemibiotrophic (*FOC*, *C. higginsianum*) fungal pathogens. Our results with *MIM773* plants, in which miR773 activity is attenuated, and with plants deficient for the miR773 target *MET2* extends previous findings that flagellin treatment results in down-regulation of miR773 and that *MIR773A* overexpression increases susceptibility to infection by the hemibiotrophic bacterial pathogen *P. syringae* (Li et al. 2010c).

MIM773 apparently mount ET/JA- and SA- dependent defense responses. Arabidopsis plants are known to deploy JA/ET-, SA-, ABA- and auxin-dependent defenses against *P. cucumerina* (Llorente et al. 2008; García-Andrade et al. 2011; Sánchez-Vallet et al. 2012; Pétriacq et al. 2016). Pathogen-induced down-regulation of miR773, and up-regulation of its target *MET2,* may thus lead to a potentiated activation of defense responses. Although we provide evidence for a function of miR773 in the regulation of ET/JA- and SA-dependent defense responses, our current understanding does not allow us to determine how miR773 and its target gene *MET2* modulate the hormone-dependent activation of defense responses.

Given that *MET2* is targeted by miR773, the question arises what role miR773-guided regulation of *MET2* expression has in immunity. DNA methyltransferases and DNA demethylases function in an antagonistically manner in controlling the overall status of DNA methylation (Deleris et al. 2016). Typically, DNA methylation represses gene expression whereas hypo-methylation is frequently associated with transcriptional up-regulation. The dynamic regulation and functional consequences of DNA methylation are, however, substantially more complex. For instance, in flowering plants there are at least five distinct classes of methylated genes, including gene body methylated (gbM) genes in which there is an enrichment of CG DNA methylation within the transcribed regions and depletion at the transcriptional start and termination sites (Bewick and Schmitz 2017). Although the function of gbM remains elusive, it was proposed that it serves to regulate splicing and gene expression (Zilberman et al. 2008; Regulski et al. 2013). To note, infection of Arabidopsis plants with *P. syringae* results in dynamic changes in DNA methylation, which in turn lead to the transcriptional activation of defense-related genes (Pavet et al. 2006; Dowen et al. 2012; Yu et al. 2013). Similarly, about half of all genes that respond to infection by the oomycete pathogen *H. arabidopsidis* (*Hpa*) are also altered in mutants with DNA methylation and gene silencing defects (López-Sánchez et al. 2016). Such mutants are also affected in pathogen resistance, but they can be either more resistant or more susceptible, depending on the pathogen lifestyle. The hyper-methylated *ros1* (*REPRESSOR OF SILENCING 1*) mutant (affected in DNA demethylation) has increased susceptibility to the biotrophic oomycete *Hpa* but enhanced resistance to the necrotrophic fungus *P. cucumerina* (López-Sánchez et al., 2016). Conversely, the hypo-methylated *nrpe1* mutant (defective in RNA-directed DNA methylation, RdDM) exhibited enhanced susceptibility to *P. cucumerina* infection but resistance to *Hpa* (López-Sánchez et al 2016).

Consistent with results reported by López-Sánchez et al. (2016), up-regulation of *MET2* through interference with miR773 activity confers resistance to *P. cucumerina*, whereas down-regulation of *MET2* expression through *MIR773* overexpression increases susceptibility to infection. These observations reinforce the interpretation that DNA methylation controls basal resistance against *P. cucumerina* in Arabidopsis. DNA methylation may exert persistent control over defense genes in the absence of infection, with infection triggering both DNA methylation and gene expression changes. DNA methylation might modulate immune responses either by modifying the activity of gene promoters and/or bodies, or by regulating the transcriptional status of transposable elements at specific loci. For instance, the methylation status of the COPIA retrotransposon has been shown to control the expression of *RPP7* (*RECOGNITION OF PERONOSPORA PARASITICA 7*), a disease resistance gene encoding an immune receptor conferring resistance to *Hpa* in Arabidopsis (Tsuchiya and Eulgem 2013).

To conclude, our results lend further support to findings that miR773a is an important component in the regulation of PTI responses. Given the absence of obvious defects in non-infected plants with altered miR773 or miR773 target activity, manipulation of miR773 or its targets may provide a new strategy for engineering crop protection.

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References

Axtell, M. J. 2013. Classification and comparison of small RNAs from plants. Annu. Rev. Plant Biol. 64:137–159.

Baldrich, P., Kakar, K., Siré, C., Moreno, A. B., Berger, A., García-chapa, M., López-moya, J. J., Riechmann, J. L., and Segundo, B. S. 2014. Small RNA profiling reveals regulation of Arabidopsis miR168 and heterochromatic siRNA415 in response to fungal elicitors. BMC Genomics. 15:1–16.

Bewick, A. J., and Schmitz, R. J. 2017. Gene body DNA methylation in plants. Curr. Opin. Plant Biol. 36:103–110.

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

Boccara, M., Sarazin, A., Thiébeauld, O., Jay, F., Voinnet, O., Navarro, L., and 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 Pathog. 10:1–16.

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.

Bravo-Ruiz, G. B., Pietro, A. D. I., and Roncero, M. I. G. 2016. Combined action of the major secreted exo- and endopolygalacturonases is required for full virulence of Fusarium oxysporum. Mol. Plant Pathol. 17:339–353.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y. Y., Sieburth, L., and Voinnet, O. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. Science (80-.). 320:1185–1190

Campo, S., Peris-Peris, C., Siré, C., Moreno, A. B., Donaire, L., Zytnicki, M., Notredame, C., Llave, C., and San Segundo, B. 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 Phytol. 199:212–227.

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

Coca, M., and San Segundo, B. 2010. AtCPK1 calcium-dependent protein kinase mediates pathogen resistance in Arabidopsis. Plant J. 63:526–540.

Cui, H., Tsuda, K., and Parker, J. E. 2015. Effector-Triggered Immunity: From Pathogen Perception to Robust Defense. Annu. Rev. Plant Biol. 66:487–511.

Deleris, A., Halter, T., and Navarro, L. 2016. DNA Methylation and Demethylation in Plant Immunity. Annu. Rev. Phytopathol. 54:579–603.

Denancé, N., Sánchez-Vallet, A., Goffner, D., and Molina, A. 2013. Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. Front. Plant Sci. 4:1–12

Dowen, R. H., Pelizzola, M., Schmitz, R. J., Lister, R., Dowen, J. M., and Nery, J. R. 2012. Widespread dynamic DNA methylation in response to biotic stress. Proc. Natl. Acad. Sci. U.S.A. 109:183–251.

Fahlgren, N., Howell, M. D., Kasschau, K. D., Chapman, E. J., Sullivan, C. M., Cumbie, J. S., and Givan, S. A. 2007. High-Throughput Sequencing of Arabidopsis microRNAs : Evidence for Frequent Birth and Death of MIRNA Genes. PLoS One. 219:1–14.

Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. 43:205–227.

Huang, J., Yang, M., and Zhang, X. 2016. The function of small RNAs in plant biotic stress response. J. Integr. 58:312–327.

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

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

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

Lee, H. J., Park, Y. J., Kwak, K. J., Kim, D., Park, J. H., Lim, J. Y., Shin, C., Yang, K.-Y., and Kang, H. 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, C., and Zhang, B. 2016. MicroRNAs in Control of Plant Development. J. Cell. Physiol. 231:303–313.

Li, S., Liu, L., Zhuang, X., Yu, Y., Liu, X., Cui, X., Ji, L., Pan, Z., Cao, X., Mo, B., Zhang, F., Raikhel, N., Jiang, L., and Chen, X. 2013. MicroRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in Arabidopsis. Cell. 153:562–574.

Li, Y., Lu, Y., Shi, Y., Wu, L., Xu, Y., Huang, F., Guo, X., Zhang, Y., Fan, J., Zhao, J., Zhang, H., Xu, P., Zhou, J., Wu, X., Wang, P., and Wang, W. 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., and Zhou, J.-M. 2010. Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. Plant Physiol. 152:2222–2231.

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., Jordá, L., and 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.

Llorente, F., Muskett, P., Sánchez-Vallet, A., López, G., Ramos, B., Sánchez-Rodríguez, C., Jordá, L., Parker, J., and Molina, A. 2008. Repression of the Auxin Response Pathway Increases Arabidopsis Susceptibility to Necrotrophic Fungi. Mol. Plant. 1:496–509.

López-Sánchez, A., Stassen, J. H., Furci, L., Smith, L. M., and Ton, J. 2016. The role of DNA (de) methylation in immune responsiveness of Arabidopsis. Plant J. 88:361–374.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-mani, B., and Ton, J. 2011. Callose Deposition: A Multifaceted Plant Defense Response. Mol. Plant-Microbe Interact. 24:183–193.

Mauch-Mani, B., and Slusarenko, A. 1993. Systemic acquired resistance in Arabidopsis thaliana induced by a predisposing infection with a pathogenic isolate of Fusarium oxysporum. Mol. Plant-Microbe Interact.7: 378-383.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J. D. G. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signalling. Science 312:436–439.

Niu, D., Lii, Y. E., Chellappan, P., Lei, L., Peralta, K., and Jiang, C. 2016. miRNA863-3p sequentially targets negative immune regulator ARLPKs and positive regulator SERRATE upon bacterial infection. Nat. Commun. 7:1–13.

O’Connell, R., Herbert, C., Sreenivasaprasad, S., Khatib, M., and Dumas, B. 2004. A Novel Arabidopsis – Colletotrichum Pathosystem for the Molecular Dissection of Plant – Fungal Interactions. Mol. Plant-Microbe Interact. 17:272–282.

Palm, M. E. 1995. Plectosporium, a new genus for Fusarium tabacinum, the anamorph of Plectosphaerella cucumerina. Mycologia. 87:397–406

Park, Y. J., Lee, H. J., Kwak, K. J., Lee, K., Hong, S. W., and Kang, H. 2014. MicroRNA400-guided Cleavage of Pentatricopeptide Repeat Protein mRNAs Renders Arabidopsis thaliana More Susceptible to Pathogenic Bacteria and Fungi. Plant Cell Physiol. 55: 1660-1668.

Pavet, V., Quintero, C., Cecchini, N. M., Rosa, A. L., and Alvarez, M. E. 2006. Arabidopsis Displays Centromeric DNA Hypomethylation and Cytological Alterations of Heterochromatin Upon Attack by Pseudomonas syringae. Mol. Plant-Microbe Intarct. 19:577–587.

Pétriacq, P., Stassen, J. H., and Ton, J. 2016. Spore density determines infection strategy by the plant pathogenic fungus Plectosphaerella cucumerina. Plant Physiol. 172:1–45.

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.

Ramírez, V., Ent, S. Van Der, García-andrade, J., Coego, A., Pieterse, C. M. J., and Vera, P. 2010. OCP3 is an important modulator of NPR1- mediated jasmonic acid-dependent induced defenses in Arabidopsis. BMC Plant Biol. 10:1–13.

Regulski, M., Lu, Z., Kendall, J., Donoghue, M. T. A., Reinders, J., Llaca, V., Deschamps, S., Smith, A., Levy, D., McCombie, W. R., Tingey, S., Rafalski, A., Hicks, J., Ware, D., and Martienssen, R. A. 2013. The maize methylome influences mRNA splice sites and reveals widespread paramutation-like switches guided by small RNA. Genome Res. 23:1651–1662.

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. G. 2011. Hormone Crosstalk in Plant Disease and Defense: More Than Just JASMONATE-SALICYLATE Antagonism. Annu. 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.

Rubio-Somoza, I., and 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., Llorente, F., Fernández, P. V., Miedes, E., Estevez, J. M., Grant, M., and Molina, A. 2012. Disruption of Abscisic Acid Signalling Constitutively Activates Arabidopsis Resistance to the Necrotrophic Fungus Plectosphaerella cucumerina. Plant Physiol. 160:2109–2124.

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

Seo, J., Wu, J., Lii, Y., Li, Y., and Jin, H. 2013. Contribution of Small RNA Pathway Components in Plant Immunity. Mol. Plant-Microbe-Interact. 26:617–625.

Shivaprasad, P. V, Chen, H., Patel, K., Bond, D. M., Santos, B. A. C. M., and Baulcombe, D. C. 2012. A MicroRNA Superfamily Regulates Nucleotide Binding Site – Leucine-Rich Repeats and Other mRNAs. Plant Cell. 24:859–874.

Soto-Suarez, M., Baldrich, P., Weigel, D., Rubio-Somoza, I., and San Segundo, B. 2017. The Arabidopsis miR396 mediates pathogen-associated molecular pattern-triggered immune responses against fungal pathogens. Sci. Rep. 7:1–14.

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

Sunkar, R., Li, Y. F., and Jagadeeswaran, G. 2012. Functions of microRNAs in plant stress responses. Tr. Plant Sci. 17:196–203.

Todesco, M., Rubio-somoza, I., Paz-ares, J., and Weigel, D. 2010. A Collection of Target Mimics for Comprehensive Analysis of MicroRNA Function in Arabidopsis thaliana. Plos Genet. 6:1–10.

Ton, J., and Mauch-Mani, B. M. 2004. b-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. D. G., and Dangl, J. L. 2006. Reactive Oxygen Species Signalling in Response to Pathogens. Plant Physiol. 141:373–378.

Tsuchiya, T., and Eulgem, T. 2013. An alternative polyadenylation mechanism coopted to the Arabidopsis RPP7 gene through intronic retrotransposon domestication. Proc. Natl. Acad. Sci. U.S.A. 110:3535–3543.

Van Loon, L. C., Rep, M., and Pieterse, C. M. J. 2006. Significance of Inducible Defense-related Proteins in Infected Plants. Annu. Rev. Phytopathol. 44:135–162.

Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E. F., and Hellens, R. P. 2007. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods. 3-12.

Vleesschauwer, D. De, Xu, J., and Höfte, M. 2014. Making sense of hormone-mediated defense networking: from rice to Arabidopsis. Front. Plant Sci. 5:1–15.

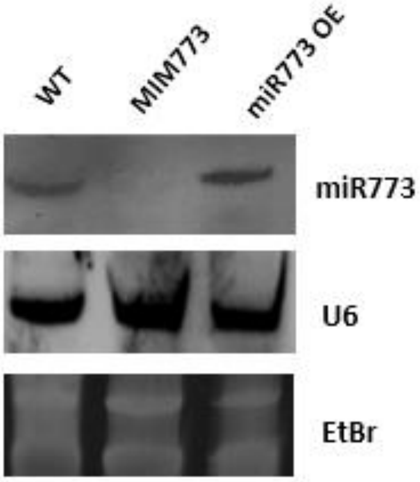
Wang, J., Schwab, R., Czech, B., Mica, E., and Weigel, D. 2008. Dual Effects of miR156-Targeted SPL Genes and CYP78A5 / KLUH on Plastochron Length and Organ Size in Arabidopsis thaliana. 20:1231–1243.

Yu, A., Lepère, G., Jay, F., Wang, J., Bapaume, L., Wang, Y., and Abraham, A. 2013. Dynamics and biological relevance of DNA demethylation in Arabidopsis antibacterial defense. Proc. Natl. Acad. Sci. U. S. A. 110:2–7.

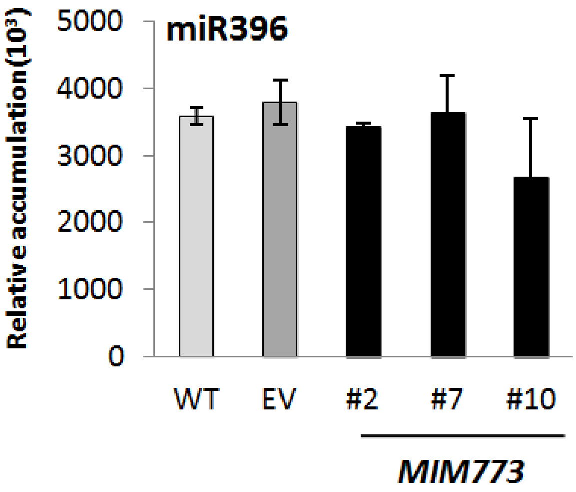
Zilberman, D., Coleman-Derr, D., Ballinger, T., and Henikoff, S. 2008. Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature. 456:125–129.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D. G., Felix, G., and Boller, T. 2004. Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428:764-767.

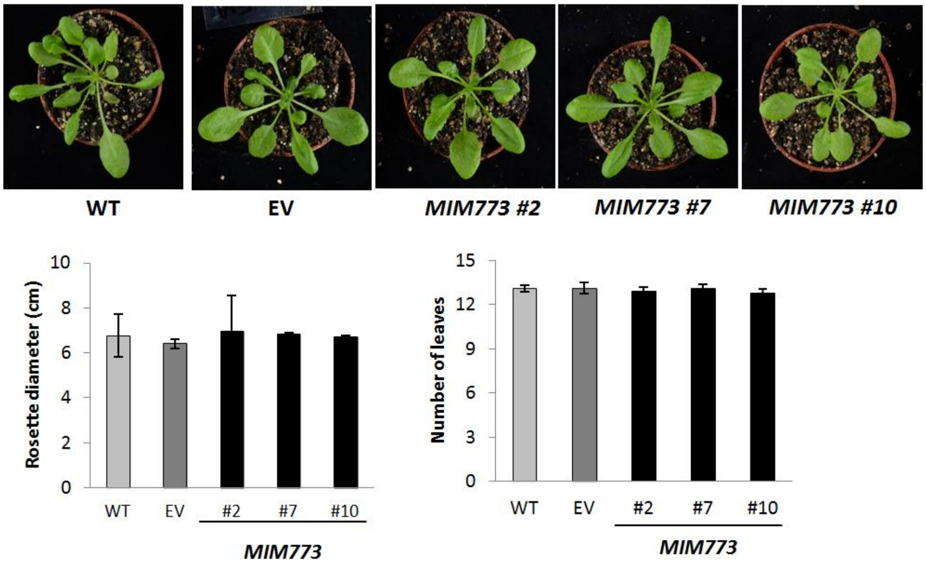
Supplemental Material



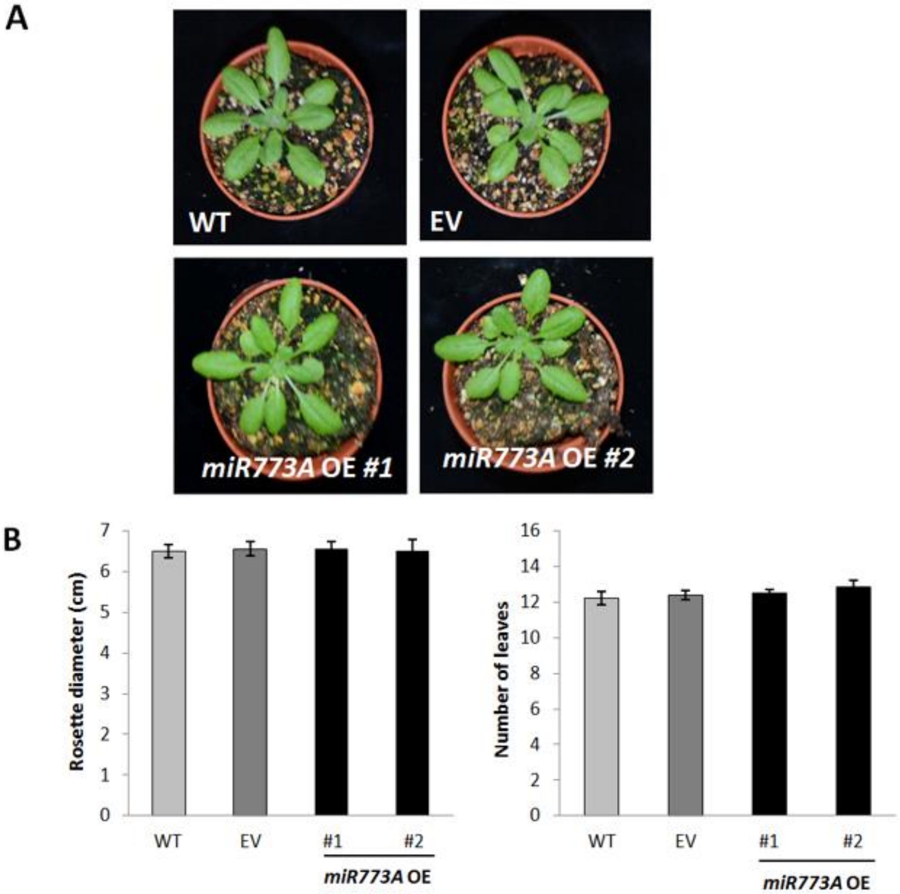
Supplemental Figure 1. Detection of mature miR773 sequences in wild-type, *MIM773* and *MIR773A* OE plants by Northern blot analysis. The small RNA fraction obtained from total RNA (600 µg) from wild-type, *MIM773* (line *MIM773*#2) and *MIR773* OE (line miR773OE#1) plants were hybridized with a 3’ end digoxigenin-labeled oligonucleotide sequence complementary to miR773. The same blot was stripped and rehybridized with the U6 probe. Lower panel, ethidium bromide staining of RNA samples.



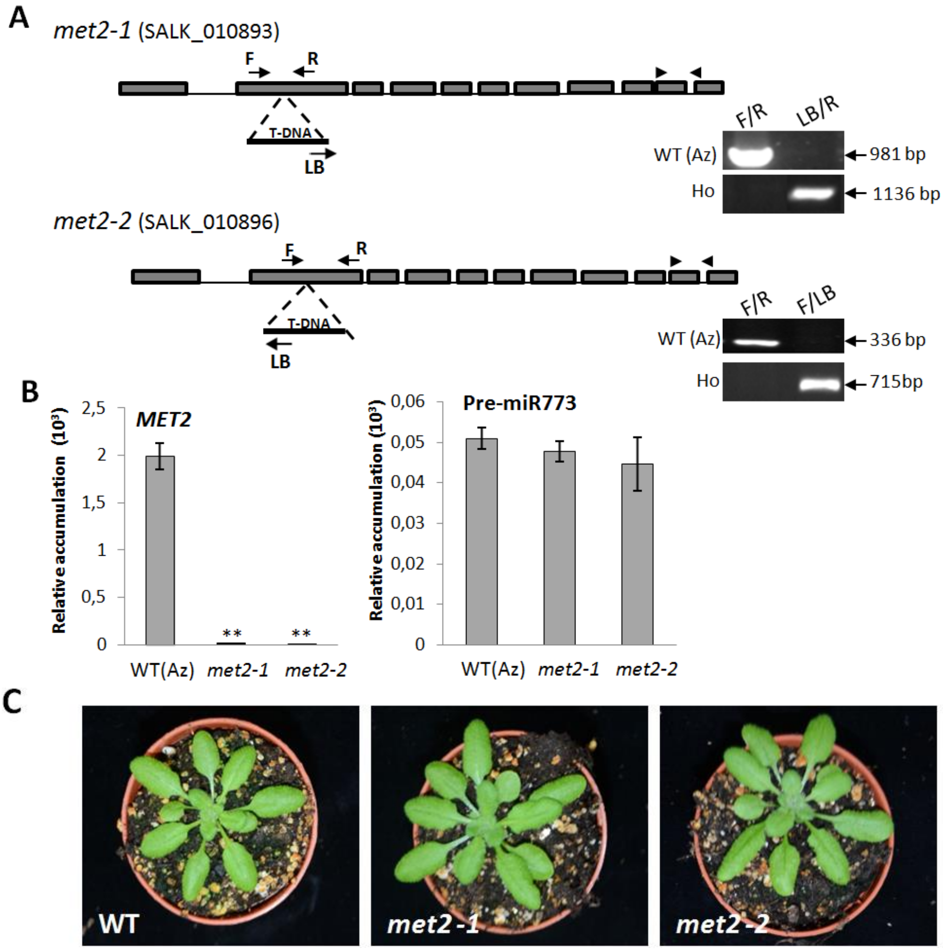
Supplemental Figure 2. Accumulation of mature miR396 sequences was determined by stem-loop reverse transcription quantitative PCR. Primers and indicated in Supplemental Table 2.



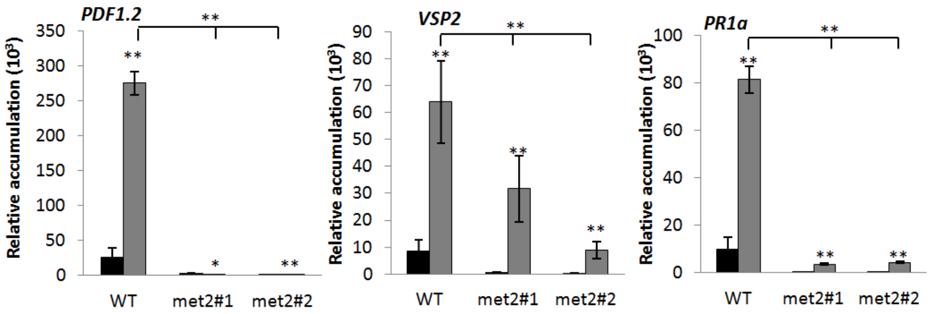
Supplemental Fig. 3. Characterization of non-infected *MIM773* lines. Plants were grown in soil for 3 weeks under neutral conditions (12h/12h, light/dark). Histograms represent the mean ± SD (ANOVA test, *, $P \leq 0.05$). Phenotype of 3 week-old *MIM773* lines. Rosette diameters and leaf number were measured by ImageJ software.



Supplemental Figure 4. Characterization of non-infected *MIR773A* OE plants. Plants were grown in soil for 3 weeks under neutral conditions (12h/12h, light/dark). Rosette diameters and leaf number were measured by ImageJ software. **A,** Rosettes of *MIR773A* OE plants. **B,** Rosette diameter and leaf number of *MIR773A* OE plants.



Supplemental Figure 5. Characterization of non-infected *met2* mutants. Plants were grown in soil for 3 weeks under neutral conditions (12h/12h, light/dark). **A,** Schematic representation of T-DNA insertion *met2* mutants identified in the SALK collection, *met2-1* (SALK_010893) and *met2-2* (SALK_010896) (genetic background Col-0). Exons and introns are indicated by black boxes and lines, respectively. Primers used for the analysis of T-DNA integration and RT-qPCR analysis are indicated by arrows and arrowheads, respectively. PCR analysis of the T-DNA integration site on genomic DNA is shown on the right. LB, left T-DNA border primer; F and R, *MET2*-specific primers; Ho, homozygous. **B**, *MET2* and pre-miR773a accumulation in rosette leaves of *met2* mutants was determined by RT-qPCR. Histogram represent the mean ± SD (ANOVA test, **, P ≤ 0.01). **C,** Rosettes of *met2* plants.



Supplemental Figure 6. Expression of defense marker genes in wild-type and *met2* plants. RT-qPCR analysis showing the accumulation of transcripts for genes that are regulated by the JA/ET-dependent (*PDF1.2*), JA-dependent (*VSP2*) and SA-dependent (*PR1a*) signalling pathways at 48 hours after inoculation with *P. cucumerina* spores. 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 12 plants per genotype. Statistical significance was determined by ANOVA (*, $P \leq 0.05$, **, $P \leq 0.01$, *P. cucumerina*-inoculated *vs* mock-inoculated; wild-type *P. cucumerina* –inoculated 48h *vs met2 P.cucumerina* –inoculated 48h).

Supplemental Table 1. *Arabidopsis thaliana* lines used in this work.

Name	**Described by**	**Ecotype**
wild-type	-	Col-0
empty vector (pGreen vector)	Todesco et al 2010	Col-0
MIM406	Todesco et al 2010	Col-0
MIM416	Todesco et al 2010	Col-0
MIM417	Todesco et al 2010	Col-0
MIM420	Todesco et al 2010	Col-0
MIM772	Todesco et al 2010	Col-0
MIM773	Todesco et al 2010	Col-0
MIM774	Todesco et al 2010	Col-0
MIM777	Todesco et al 2010	Col-0
MIM783	Todesco et al 2010	Col-0
MIM841	Todesco et al 2010	Col-0
miR773A OE (35Sprom::MIR773A)	Present work	Col-0
empty vector (pCAMBIA1300)	Present work	Col-0
met2-1	SALK_010893	Col-0
met2-2	SALK_010896	Col-0

Supplemental Table 2. Sequences of oligonucleotides used.

Oligo ID	*Accesion number*		Sequence (5'-3')
For expression analysis			
β-Tubulin 2	*At5g62690*	*Frw*	TGTTCAGGCGAGTGAGTGAG
		Rev	ATGTTGCTCTCCGCTTCTGT
miR773 Stem loop		*RT*	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGACA
		Frw	GGCGGCTTTGCTTCCAGCTTT
miR396 Stem loop		*RT*	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGTTC
		Frw	GGCGGCTTCCACAGCTTTCTT
Pre-miR773a	*At1g35501*	*Frw*	TGATTGCAGAAGTCCATCGA
		Rev	GAGACAAAAGCTGGAAGCAAA
MET2	*At4g14140*	*Frw*	ACCTGCCGGACGAAAATGTG
		Rev	TCGTAGCTATCCGGAAACCC
PDF1.2	*At2g26020*	*Frw*	CAACAATGGTGGAAGCACAG
		Rev	CTTGCATGCATTGCTGTTTC
VSP2	*At5g24770*	*Frw*	CTCGTCGATTCGAAAACCAT
		Rev	TTCTGCAGTTGGCGTAGTTG
PR1	*At2g14610*	*Frw*	GATGTGCCAAAGTGAGGTGTAA
		Rev	GGCTTCTCGTTCACATAATTCC
NPR1	*At1g64280*	*Frw*	CCGGAAGAGCTTGTTAAAGAGA
		Rev	ATCCGAGTCAAGTGCCTTATGT
ACS1	*At3g61510*	*Frw*	ACGCTTTTCTCGTCCCTACTC
		Rev	GGCCTTAAGGTACGCTGATTC
ACO2	*At1g62380*	*Frw*	AGGAAACAGGATGTCGGTTG
		Rev	CGGAATCTTTCTCGACAAGC
For fungal DNA quantification			
P.cucumerina_tubulin		*Frw*	CAAGTAtGTTCCCCGAGCCGT
		Rev	GAAGAGCTGACCGAAGGGACC
FOC_chsV		*Frw*	ACAGCTCCAACGAACTCTCTT
		Rev	GGAGGTACTTGGTCAtGTCGT
Ch_ITS2		*Frw*	AAAGGTAGTGGCGGACCCTC
		Rev	GGCAAGAGTCCCTCCGGAt
Ubiquitin21	*At5g25760*	*Frw*	AAAGGACCTTCGGAGACTCCTTACG
		Rev	GGTCAAGAAtCGAACTTGAGGAGGTT
For *met2* genotyping			
MET2 #1		*Frw*	CTTCTGGTGGTTCAAGCAGGTT
		Rev	GCTCCTCTTCCTCCACATTTTCAGCATTA
MET2 #2		*Frw*	CTCAATGATTCAAAGACAGGATTTC
		Rev	CGGAGGGAGAATATATG
T-DNA LB		*Frw*	ACCGCCTGGCCCTGAGAGAGTT
For construct preparation			
MIR773A	*At1g35501*	*Frw **	CGC**GGTACC**CGGATGTGAAAGCAAACAAAAAAATGAAGCTG
		*Rev**	CGC**TCTAGA**CGTCGTAAATTCTCCAGACCTCCAAAACTC
For miR773a *in situ* hybridization			
ath-miR773-LNA			GAGACAAAAGCTGGAAGCAAA
For miR773a Northern Blot			
ath-miR773-DIG			GAGACAAAAGCTGGAAGCAAA
U6-DIG			ATTTGGACCATTTCTCGATTTGT

* In bold, recognition sites for the restriction enzymes KpnI (GGTACC) and XbaI (TCTAGA)

General Discussion

General discussion

Studies in different plant species have demonstrated the crucial role of miRNAs in a wide range of plant developmental processes, such as organ polarity and morphogenesis, flowering, shoot and root development, and hormone signaling, among others. There are also reports indicating that miRNAs respond to different types of abiotic and biotic stress. However, our knowledge on miRNAs involved in plant immunity is scarce. Most research on plant miRNAs has been conducted in the model dicotyledonous plant *Arabidopsis thaliana*.

A significant progress has been made during the last years for the identification of miRNAs in rice. The relevance of distinct rice miRNAs in controlling traits of agronomic importance, such as tiller growth, early flowering, panicle and grain production, is well demonstrated. These studies focused on the characterization of the miRNA population in different tissues during development of the rice plant. In this Ph.D Thesis, we investigated miRNAs contributing to rice and Arabidopsis immunity, the model species for studies on functional genomics in monocot and dicot plants. In rice, we also investigated the contribution of DCL1, a key component in miRNA biogenesis, in the rice response to infection by fungal pathogens.

The results obtained in this Ph.D Thesis (**Chapter I**) demonstrated that a polycistronic miRNA from rice, miR166k-166h, plays an important function in rice immunity against fungal pathogens. For the functional characterization of this polycistronic miRNAs, bioinformatic analyses were initially carried out for prediction of the miRNA precursor structure and putative target genes. Upon pathogen challenge, there is an increase in the accumulation of precursor miR166k-166h transcripts, as well as mature miRNAs derived from this precursor. Similarly, treatment with fungal elicitors increase miR166k-166h transcript levels. These observations were already indicative of a possible involvement of this polycistronic miRNA in the rice defense response, particularly in PTI. Definitive evidence on the involvement of miR166k-166h in rice immunity came from disease resistance experiments in miR166k-166h activation mutants, these plants exhibiting enhanced resistance to pathogen infection. The phenotype of disease resistance in these plants is associated to stronger activation of defense gene expression, namely *PR1a* and *PBZ1* (markers of the induction of defense responses in rice). An interesting finding of this study was that, in addition to mediate regulation of *Hox* genes in rice, the miR166k-166h also mediates regulation of two novel target genes for miR166, *EIN2* and *ferredoxin-nitrite reductase*. Our study also revealed that *EIN2* and *ferredoxin-nitrite reductase* are the targets for the star strands of miR166k and miR166h (targeting *EIN2* and *ferredoxin-nitrite reductase*, respectively). Based on gene expression analysis of components of the ethylene signaling pathway, a miR166k-166h-mediated regulation of ethylene-dependent defense responses in miR166k-166h activation mutants is proposed. Further studies are, however, needed to determine ethylene levels in these plants during normal growth conditions as well as during pathogen infection.

During the course of the work for this Ph.D Thesis, two additional polycistronic miRNAs from rice have been studied. They are: miR1861b-1861c and miR5534a-5534b. Compared to miR166k-166h, these studies are in a less advanced status and are summarized in Annex I. As in the case of miR166k-166h, miR1861b-1861c and miR5534a-5534b function as positive regulators of rice immunity.

The discovery of polycistronic miRNA precursors in plants is very recent and the functionality of this organization is still in debate. Even so, polycistronic precursors can control gene expression regulation of several biological processes (for instance defense and development), allowing multiple miRNAs being expressed in a spatio-temporal maneer. In the case of plant immunity, polycistronic miRNAs could regulate simultaneously diverse genes involved in plant defense responses.

Other studies carried out during the course of this Ph.D Thesis (not included in this document) relate to the functional characterization of small RNAs from rice representing novel miRNA candidates, as well as two novel miRNAs recently identified in our group (miR11340, miR11343) (Baldrich et al. 2015). Thus, based on the information gained by deep sequencing of small RNA libraries from rice tissues treated, or not, with *M. oryzae* elicitors, a set of small RNAs were identified as novel miRNA candidates. They were: miR-8, miR-13, miR-55, miR-57 and miR-103 (hypheneted names are used to distinguish these sequences from real miRNAs). I generated transgenic rice plants overexpressing each of these miRNA candidates. The miR-8 overexpressor lines showed enhanced susceptibility to *M. oryzae* infection as revealed by visual inspection of disease symptoms and higher accumulation of fungal biomass than wild-type plants. Bioinformatic analysis revealed that a *NBS-LRR* gene can be a putative target for miR-8, and its expression was found to be down-regulated in miR-8 overexpressor lines. As for the other miRNA candidates, their overexpression was found to confer enhanced resistance to infection by the rice blast fungus *M. oryzae*. In the future, we will investigate the accumulation of these miRNA candidates in rice mutants impaired in small RNA biogenesis genes (*dcl1, dcl3, dcl4, rdr2* mutants), that are being characterized in our group. This will allow us to know whether these small RNA sequences are real miRNAs for their official annotation in the miRBase registry. Regarding the two recently annotated miRNAs, miR11340 and miR11343, the overexpression of each one of these miRNAs has been shown to confer resistance to infection by *M. oryzae*. Furthermore, by using the modified 5' RACE technique, the *chitin-inducible gibberellin-responsive protein 2* gene has been validated as the target gene for miR11340. Finally, CRISPR/Cas9 vectors have been prepared for targeted genome editing of miR11340-5p and its target gene, which will be used for rice transformation in the near future.

Concerning the implication of *OsDCL1a* in plant immunity (**Chapter II**), by using *DCL1a* activation lines (*dcl1a-Ac*), we demonstrated that a high expression level of *OsDCL1a* results in susceptibility to infection by hemibiotrophic (*M. oryzae*) and necrotrophic (*Fusarium fujikuroi*) fungal pathogens, ergo, *DCL1a* activation negatively regulates rice immunity. To infer how *DCL1a* regulates rice immunity against *M. oryzae*, a foliar pathogen, we obtained the mRNA and small RNA transcriptomes of *dcl1a-Ac* leaves. Interestingly, the mRNA transcriptome revealed that genes involved in two processes were severely affected by *OsDCL1a* activation. Thus, genes involved in redox homeostasis and diterpenoid phytoalexin biosynthesis genes were down-regulated in *dcl1a-Ac* mutant plants. Consistent with the observed mis-regulation of genes involved in detoxification of reactive oxygen species, the *dcl1a-Ac* mutant plants are more sensitive to oxidative stress induced by methyl viologen. Also, knowing that diterpene phytoalexins have antifungal activity against *M. oryzae*, it is expected that a reduced expression of diterpene phytoalexin genes during *M. oryzae* infection is accompanied by lower levels of phytoalexins. This, in turn, might contribute to disease susceptibility. Analysis of phytoalexin content in *dcl1a-Ac* mutant plants is, however, needed to confirm this aspect. Furthermore, *DCL1a* activation results in important alterations in the miRNAome. The group of miRNAs differentially accumulating in *dcl1-Ac* mutant plants not only included up-regulated miRNAs, but also down-regulated miRNAs. Among the down-regulated miRNAs, there were miR393 and miR398 which are known to be positive regulators of plant immunity (Navarro et al. 2006; Li et al. 2014a). The down-regulation of some miRNA can be explained by several reasons. One of them is the temporal expression pattern of genes involved in miRNA biogenesis other than *OsDCL1a*. Also, down-regulation of some miRNAs might be due to a lack of miRNA stability (which is also dependent on miRNA modifications), binding of miRNAs to AGO (which protects miRNAs from degradation), or sequestration by target mimic RNAs. An additional reason exists on auto-regulatory feedback loops between miRNA and target genes, in which target genes can control the level of a miRNA in addition to being regulated by it.

Chapter III focused on the functional characterization of miR773 in Arabidopsis, this particular miRNA targeting *Methyltransferase 2* (*MET2*). By examining Arabidopsis plants in which the activity of miR773 is blocked through the expression of a miR773 target mimic (*MIM773* plants), miR773 overexpressor plants, and *met2* mutant plants, it is concluded that miR773 plays a negative role in controlling Arabidopsis immunity. miR773 functions in the Arabidopsis response to infection by necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Fusarium oxysporum* f. sp. *conglutinans, Colletotrichum higginsianum*) fungal pathogens. *MIM773* plants exhibited resistance to infection by these pathogens, this phenotype being associated to stronger PTI. Upon pathogen challenge, *MIM773* plants accumulated higher levels of callose and reactive oxygen species than wild-type plants. These plants also exhibited stronger induction of defense gene expression in response to pathogen infection. Knowing that miR773 targets *MET2*, a DNA methyl transferase, the results obtained in this work reinforce the importance of DNA methylation in plant immunity. In this respect, changes in DNA methylation have been already associated to Arabidopsis resistance to necrotrophic fungi (López-Sánchez et al. 2016). The exact mechanisms by which a miR773-mediated regulation of *MET2* has an impact on DNA methylation in Arabidopsis deserves further investigation.

Collectively, results obtained in this work support that miRNAs, and miRNA biogenesis components (e.g. DCL1) can be considered as an integral part of the plant response to infection by fungal pathogens. Disease resistant plants can be obtained by modulating the expression of miRNAs for which a role in plant immunity is known, or their corresponding target genes. Since pathogen infection is one of the main causes of crop losses worldwide, unravelling miRNA-mediated mechanisms involved in disease resistance may lead to biotechnological applications for the development of appropriate strategies for crop protection.

Conclusions

Conclusions

1. Expression of the rice polycistronic *MIR166k-166h* is induced by infection with the blast fungus *Magnaporthe oryzae* and treatment with fungal elicitors, suggesting that miR66k-166h plays a role in PTI in rice.

2. The miR166k-166h precursor encodes four mature miR166 sequences: miR166k, miR166k*, miR166h and miR166h*. All four miR166 sequences in miR166k-166h are functional.

3. *MIR166k-166h* activation confers resistance to infection by *M. oryzae* indicating that this polycistronic miRNA functions as a positive regulator of rice immunity. Rice mutants in which *MIR166k-166h* is activated show a strong induction of defense gene expression during fungal infection.

4. Two novel targets genes have been identified for miR166: *Ethylene insensitive 2* (*EIN2*) and *Ferredoxin-nitrite reductase,* which are the target genes for miR166k* and miR166h*, respectively. The regulatory role of miR166k-166h in rice immunity, most probably, results from a miR166k*-mediated modulation of the ethylene signaling pathway.

5. In rice, *OsDCL1a* activation increases susceptibility to infection by the fungal pathogens *Magnaporthe oryzae* and *Fusarium fujikuroi.* Pathogen-induced defense gene expression is compromised in activation-tagged *dcl1a* mutant plants, supporting that *OsDCL1a* functions as a negative regulator in rice immunity.

6. *OsDCL1a* activation causes misregulation of genes involved in detoxification of reactive oxygen species and accumulation of the superoxide anion $O_2^{\cdot -}$ in rice leaves. *OsDCL1a* activation also results in down-regulation of diterpenoid phytoalexin biosynthesis genes. Whether induction of *OsDCL1* expression is accompanied by a lower accumulation of diterpenoid phytoalexins during pathogen infection remains to be determined.

7. Important alterations occur in the rice miRNAome as a consequence of *OsDCL1a* activation, including both up-regulation and down-regulation of miRNAs. Presumably, *OsDCL1a*-mediated alterations in the accumulation of rice miRNAs, and their corresponding target genes, have an impact on disease resistance. Together, results here presented support that *OsDCL1a* plays an important role in rice immunity.

8. In Arabidopsis, interference with miR773 by target mimics (*MIM773* plants), and concomitant up-regulation of the miR773 target gene *METHYLTRANSFERASE 2* (*met2*), increases resistance to infection by necrotrophic (*Plectosphaerella cucumerina*) and hemibiotrophic (*Fusarium oxysporum*, *Colletotrichum higginsianum*) fungal pathogens. Resistance to infection by *P. cucumerina* in *MIM773* plants is associated with a stronger expression of basal defense responses upon pathogen infection, including higher accumulation of H_2O_2 and callose, and superactivation of defense gene expression. Conversely, miR773 overexpression and *MET2* silencing results in enhanced susceptibility to pathogen infection.

9. miR773 accumulation decreases not only during pathogen infection but also in response to treatment with fungal elicitors suggesting that miR773 is a component of PTI.

10. The miR773 target gene *MET2* encodes a DNA methyltransferase. A miR773-mediated regulation of *MET2* expression might regulate the methylation status of the host DNA for the control of immune responses.

Bibliography

Bibliography

Abbruscato, P., Nepusz, T., Mizzi, L., Del Corvo, M., Morandini, P., Fumasoni, I., Michel, C., Paccanaro, A., Guiderdoni, E., Schaffrath, U., Morel, J. B., Piffanelli, P., and Faivre-Rampant, O. 2012. OsWRKY22, a monocot wrky gene, plays a role in the resistance response to blast. Mol. Plant Pathol. 13:828–841

Achkar, N. P., Cambiagno, D. A., and Manavella, P. A. 2016. miRNA Biogenesis: A Dynamic Pathway. Trends Plant Sci. xx:1–11

Addo-Quaye, C., Snyder, J. A., Park, Y. B., Li, Y., Sunkar, R., and Axtell, M. J. 2009. Sliced microRNA targets and precise loop-first processing of MIR319 hairpins revealed by analysis of the Physcomitrella patens degradome. RNA. 15:2112–2121

Agorio, A., and Vera, P. 2007. ARGONAUTE4 is required for resistance to Pseudomonas syringae in Arabidopsis. Plant Cell. 19:3778–3790

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

Akagi, A., Fukushima, S., Okada, K., Jiang, C. J., Yoshida, R., Nakayama, A., Shimono, M., Sugano, S., Yamane, H., and Takatsuji, H. 2014. WRKY45-dependent priming of diterpenoid phytoalexin biosynthesis in rice and the role of cytokinin in triggering the reaction. Plant Mol. Biol. 86:171–183

Altuvia, Y., Landgraf, P., Lithwick, G., Elefant, N., Aravin, A., Brownstein, M. J., Tuschl, T., and Margalit, H. 2005. Clustering and conservation patterns of human microRNAs. Nucleic Acids Res. 33:2697–2706

Alvarez-Venegas, R., Abdallat, A. Al, Alfano, J. R., and Avramova, Z. 2007. Epigenetic Control of a Transcription Factor at the Cross Section of Two Antagonistic Pathways. Epigenetics. 2:106–117

Aranega-Bou, P., Leyva, M. D. O., Finiti, I., and García-agustín, P. 2014. Priming of plant resistance by natural compounds. Hexanoic acid as a model. Front. Plant Sci. 5:1–12

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

Ashikawa, I., Hayashi, N., Yamane, H., Kanamori, H., Wu, J., Matsumoto, T., Ono, K., and Yano, M. 2008. Two Adjacent Nucleotide-Binding Site–

Leucine-Rich Repeat Class Genes Are Required to Confer Pikm -Specific Rice Blast Resistance. Genetics. 180:2267–2276

Ausin, I., Greenberg, M. V. C., Li, C. F., and Jacobsen, S. E. 2012. The splicing factor SR45 affects the RNA-directed DNA methylation pathway in Arabidopsis. © 2012 Landes Bioscience. 7:29–33

Axtell, M. J. 2013. Classification and comparison of small RNAs from plants. Annu Rev Plant Biol. 64:137–159

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

Baldrich, P., Hsing, Y. C., and Segundo, B. S. 2016. Genome-Wide Analysis of Polycistronic MicroRNAs in Cultivated and Wild Rice. GBE. 8:1104–1114

Ballini, E., Morel, J., Droc, G., Price, A., Courtois, B., Notteghem, J., and Tharreau, D. 2008. A Genome-Wide Meta-Analysis of Rice Blast Resistance Genes and Quantitative Trait Loci Provides New Insights into Partial and Complete Resistance. Mol. plant microbe Interact. 21:859–868

Barik, S., Sarkardas, S., Singh, A., Gautam, V., Kumar, P., Majee, M., and Sarkar, A. K. 2014. Genomics Phylogenetic analysis reveals conservation and diversi fi cation of micro RNA166 genes among diverse plant species. Genomics. 103:114–121

Bartel, D. P., Lee, R., and Feinbaum, R. 2004. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function Genomics: The miRNA. Genes. 116:281–297

Bian, H., Xie, Y., Guo, F., Han, N., Ma, S., Zeng, Z., Wang, J., Yang, Y., and Zhu, M. 2012. Distinctive expression patterns and roles of the miRNA393 ⁄ TIR1 homolog module in regulating flag leaf inclination and primary and crown root growth in rice (Oryza sativa). New Phytol. 196:149–161

Blevins, T., Rajeswaran, R., Shivaprasad, P. V., Beknazariants, D., Si-Ammour, A., Park, H. S., Vazquez, F., Robertson, D., Meins, F., Hohn, T., and Pooggin, M. M. 2006. Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. Nucleic Acids Res. 34:6233–6246

Boccara, M., Sarazin, A., Thiébeauld, O., Jay, F., Voinnet, O., Navarro, L., and 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 Pathog. 10:1–16

Bologna, N. G., and Voinnet, O. 2014. The Diversity, Biogenesis, and Activities of Endogenous Silencing Small RNAs in Arabidopsis. Annu Rev Plant Biol. 65:473–503

Brien, J. A. O., Daudi, A., Butt, V. S., and Bolwell, G. P. 2012. Reactive oxygen species and their role in plant defence and cell wall metabolism. Planta. 236:765–779

Brodersen, P., and Voinnet, O. 2006. The diversity of RNA silencing pathways in plants. Trends Genet. 22:268–280

Bryan, G. T., Wu, K., Farrall, L., Jia, Y., Hershey, H. P., Mcadams, S. A., Faulk, K. N., Donaldson, G. K., Tarchini, R., and Valent, B. 2000. A Single Amino Acid Difference Distinguishes Resistant and Susceptible Alleles of the Rice Blast Resistance Gene Pi-ta. Plant Cell. 12:2033–2045

Calviño, M., and Messing, J. 2013. Discovery of MicroRNA169 Gene Copies in Genomes of Flowering Plants through Positional Informationnd messing 2013. Genome Biol. Evol. 5:402–417

Campo, S., Peris-Peris, C., Siré, C., Moreno, A. B., Donaire, L., Zytnicki, M., Notredame, C., Llave, C., and San Segundo, B. 2013b. 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 Phytol. 199:212–227

Campos-Soriano, L., Valè, G., Lupotto, E., and San Segundo, B. 2013. Investigation of rice blast development in susceptible and resistant rice cultivars using a gfp- expressing Magnaporthe oryzae isolate. Plant Pathol. 9:1030–1037

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

Chen, L., Luan, Y., and Zhai, J. 2015. Sp-miR396a-5p acts as a stress-responsive genes regulator by conferring tolerance to abiotic stresses and susceptibility to Phytophthora nicotianae infection in transgenic tobacco. Plant Cell Rep. 34:2013–2025

Chen, X. 2004. A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science (80-.). 303:2022–2025

Chen, X., Shang, J., Chen, D., Lei, C., Zou, Y., Zhai, W., Liu, G., Xu, J., Cao, G., Ma, B., Wang, Y., Zhao, X., Li, S., and Zhu, L. 2006. A B-lectin receptor kinase gene conferring rice blast resistance. Plant J. 46:794–804

Cheng, H., Li, H., Deng, Y., Xiao, J., Li, X., and Wang, S. 2015. The WRKY45-2—WRKY13—WRKY42 Transcriptional Regulatory Cascade Is Required for Rice Resistance to Fungal Pathogen. Plant Physiol. 167:pp.114.256016

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

Choi, H. W., and Klessig, D. F. 2016. DAMPs , MAMPs , and NAMPs in plant innate immunity. BMC Plant Biol. 16:1–10

Chorostecki, U., Moro, B., Rojas, A. L. M., Debernardi, J. M., Schapire, A. L., Notredame, C., and Palatnik, J. 2017. Evolutionary Footprints Reveal Insights into Plant MicroRNA Biogenesis. Plant Cell. :doi:10.1105/tpc.17.00272

Clough, S. J., and Bent, A. F. 1998. Floral dipping: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. plant J. 16:735–743

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M.-A., Pieterse, C. M. J., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L., and Mauch-Mani, B. 2006. Priming: Getting Ready for Battle. Mol. Plant-Microbe Interact. 19:1062–1071

Cottyn, B., VanOutryve, M., Cerez, M., DeCleene, M., Swings, J., Mew, T., VanOutryve, M., Barroga, J., Swings, J., and Mew, T. 1996. Bacterial diseases of rice.1. Pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. Plant Dis. 80:438–445

Couch, B. C., and Kohn, L. M. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, Magnaporthe oryzae, from M. grisea. Mycologia. 94:683–693

Courtois, C., Besson, A., Dahan, J., Bourque, S., Dobrowolska, G., Pugin, A., and Wendehenne, D. 2008. Nitric oxide signalling in plants: interplays with Ca 2 + and protein kinases. J. Exp. Bot. 59:155–163

Cui, H., Tsuda, K., and Parker, J. E. 2015. Effector-Triggered Immunity: From Pathogen Perception to Robust Defense. Annu. Rev. Plant Biol. 66:487–

511

Cuperus, J. T., Fahlgren, N., and Carrington, J. C. 2011. Evolution and Functional Diversification of MIRNA Genes. Source Plant Cell. 23:431–442

Curaba, J., Singh, M. B., and Bhalla, P. L. 2014. MiRNAs in the crosstalk between phytohormone signalling pathways. J. Exp. Bot. 65:1425–1438

Dawe, D., Pandey, S., and Nelson, A. 2010. Rice in the Global Economy: Strategic Research and Policy Issues for Food Security. Los Baños, CA: Philippines: International Rice Research Center.

Deleris, A., Halter, T., and Navarro, L. 2016. DNA Methylation and Demethylation in Plant Immunity. Annu. Rev. Phytopathol. 54:579–603

Denancé, N., Sánchez-Vallet, A., Goffner, D., and Molina, A. 2013. Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. Front. Plant Sci. 4:1–12

Deslandes, L., Olivier, J., Theulieres, F., Hirsch, J., Feng, D. X., Bittner-Eddy, P., Beynon, J., and Marco, Y. 2002. Resistance to Ralstonia solanacearum in Arabidopsis thaliana is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. Proc. Natl. Acad. Sci. U. S. A. 99:2404–9

Ding, B., and Wang, G.L. 2015. Chromatin versus pathogens: the function of epigenetics in plant immunity. Front. Plant Sci. 6:1–8

Dogara, A. M., and Jumare, A. I. 2014. Origin, Distribution and Heading date in Cultivated Rice. Int. J. plant Biol. Res. 2:2–6

Du, P., Wu, J., Zhang, J., Zhao, S., Zheng, H., Gao, G., Wei, L., and Li, Y. 2011. Viral infection induces expression of novel phased microRNAs from conserved cellular microRNA precursors. PLoS Pathog. 7:1–15

Dunoyer, P., Himber, C., and Voinnet, O. 2006. Induction, suppression and requirement of RNA silencing pathways in virulent Agrobacterium tumefaciens infections. Nat. Genet. 38:258–63

Ellendorff, U., Fradin, E. F., De Jonge, R., and Thomma, B. P. H. J. 2009. RNA silencing is required for Arabidopsis defence against Verticillium wilt disease. J. Exp. Bot. 60:591–602

Endo, M., Mikami, M., and Toki, S. 2015. Multigene knockout utilizing off-target mutations of the CRISPR/cas9 system in rice. Plant Cell Physiol. 56:41–47

Endo, Y., Iwakawa, H., and Tomari, Y. 2013. Arabidopsis ARGONAUTE7 selects miR390 through multiple checkpoints during RISC assembly. EMBO Rep.1-7

Fahlgren, N., Howell, M. D., Kasschau, K. D., Chapman, E. J., Sullivan, C. M., Cumbie, J. S., and Givan, S. A. 2007. High-Throughput Sequencing of Arabidopsis microRNAs: Evidence for Frequent Birth and Death of MIRNA Genes. PLoS One. 219:1–14

FAOSTAT 2016. faostat.fao.org

Fei, Q., Yang, L., Liang, W., Zhang, D., and Meyers, B. C. 2016a. Dynamic changes of small RNAs in rice spikelet development reveal specialized reproductive phasiRNA pathways. J. Exp. Bot. 67:6037–6049

Ferrari, S., Savatin, D. V, Sicilia, F., Gramegna, G., Cervone, F., and Lorenzo, G. De. 2013. Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. Front. Plant Sci. 4:1–9

Franco-Zorrilla, J. M., Valli, A., Todesco, M., Mateos, I., Puga, M. I., Rubio-Somoza, I., Leyva, A., Weigel, D., García, J. A., and Paz-Ares, J. 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. Nat. Genet. 39:1033–1037

Fukuoka, S., Saka, N., Koga, H., Ono, K., Shimizu, T., Ebana, K., Hayashi, N., Takahashi, A., Hirochika, H., Okuno, K., and Yano, M. 2009. Loss of Function of a Proline-Containing Protein Confers Durable Disease Resistance in Rice. Science (80-.). 25:998–1002

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

Gasciolli, V., Mallory, A. C., Bartel, D. P., and Vaucheret, H. 2005. Partially Redundant Functions of Arabidopsis DICER-like Enzymes and a Role for DCL4 in Producing trans -Acting siRNAs. Curr. Biol. 15:1494–1500

Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. 43:205–27

Goff, S. A., Ricke, D., Lan, T., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Varma, H., Hadley, D., Hutchison, D., Martin, C., Katagiri, F., Lange, B. M., Moughamer, T., Xia, Y., Budworth, P., Zhong, J., Miguel, T., Paszkowski, U., Zhang, S., Colbert, M., Sun, W., Chen, L., Cooper, B., Park, S., Wood, T. C., Mao, L., Quail, P., Wing, R., Dean, R., Yu, Y., Zharkikh, A., Shen, R., Sahasrabudhe, S., Thomas, A., Cannings, R., Gutin, A., Pruss, D., Reid, J., Tavtigian, S., Mitchell, J., Eldredge, G., Scholl, T., Miller, R. M., Bhatnagar, S., Adey, N., Rubano, T., Tusneem, N., Robinson, R., Feldhaus, J., Macalma, T., Oliphant, A., and Briggs, S. 2002. A Draft Sequence of the Rice Genome (Oryza sativa L. ssp. japonica). Science (80-.). 296:92–100

Goto, M. 1979. Bacterial Foot Rot of Rice Caused by a Strain of Erwinia chrysanthemi. Phytopathology. 69:213–216

Grayer, R. J., and Kokubun, T. 2001. Plant - fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants. Phytochemistry. 56:253–263

Hamilton, A. J., and Baulcombe, D. C. 1999. A Species of Small Antisense RNA in Posttranscriptional Gene Silencing in Plants. Science (80-.). 286:1997–2000

Hasegawa, M., Mitsuhara, I., Seo, S., Imai, T., Koga, J., Okada, K., Yamane, H., and Ohashi, Y. 2010. Phytoalexin Accumulation in the Interaction Between Rice and the Blast Fungus. Mol. Plant-Microbe Interact. 23:1000–1011

Hayashi, K., and Yoshida, H. 2009. Refunctionalization of the ancient rice blast disease resistance gene Pit by the recruitment of a retrotransposon as a promoter. plant J. 57:413–425

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., Lu, L., and Zhang, X. 2016. Diverse Functions of Small RNAs in Different Plant – Pathogen Communications. Funct. Int. Genom. 7:1–17

Huang, X., Kurata, N., Wei, X., Wang, Z., Wang, A., Zhao, Q., Zhao, Y., Liu, K., Dong, G., Zhan, Q., Li, C., Fujiyama, A., Toyoda, A., Lu, T., Feng, Q., Qian, Q., and Li, J. 2012. A map of rice genome variation reveals the origin of cultivated rice. Nature. 490:497–501

Jacob, F., Vernaldi, S., and Maekawa, T. 2013. Evolution and conservation of plant NLR functions. Front. Immunol. 4:1–16

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

Jakob, K., Goss, E. M., Araki, H., Van, T., Kreitman, M., and Bergelson, J. 2002.

Pseudomonas viridiflava and P. syringae--natural pathogens of Arabidopsis thaliana. Mol. Plant. Microbe. Interact. 15:1195–1203

Jeworutzki, E., Roelfsema, M. R. G., Anschütz, U., Krol, E., Elzenga, J. T. M., Felix, G., Boller, T., Hedrich, R., and Becker, D. 2010. Early signaling through the arabidopsis pattern recognition receptors FLS2 and EFR involves Ca2+-associated opening of plasma membrane anion channels. Plant J. 62:367–378

Jiang, D., Jiang, D., Yin, C., Yu, A., Zhou, X., Liang, W., Yuan, Z., and Xu, Y. 2006. Duplication and expression analysis of multicopy miRNA gene family members in Arabidopsis and rice. Cell Res. 16:507–518

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

Jung, H. W., Tschaplinski, T. J., Wang, L., Glazebrook, J., and Greenberg, J. T. 2009. Priming in Systemic Plant Immunity. Science (80-.). 324:89–92

Kanno, Y., Otomo, K., Kenmoku, H., Mitsuhashi, W., Yamane, H., Oikawa, H., Toshima, H., Matsuoka, M., Sassa, T., Toyomasu, T., Anno, Y. K., Tomo, K. O., Enmoku, H. K., Itsuhashi, W. M., Amane, H. Y., Ikawa, H. O., Oshima, H. T., Atsuoka, M. M., Assa, T. S., and Oyomasu, T. T. 2006. Characterization of a Rice Gene Family Encoding Type-A Diterpene Cyclases Characterization of a Rice Gene Family Encoding Type-A Diterpene Cyclases. Biosci. Biotechnol. Biochem. 70:1702–1710

Kapoor, M., Arora, R., Lama, T., Nijhawan, A., Khurana, J. P., Tyagi, A. K., and Kapoor, S. 2008. Genome-wide identification, organization and phylogenetic analysis of Dicer-like, Argonaute and RNA-dependent RNA Polymerase gene families and their expression analysis during reproductive development and stress in rice. BMC Genomics. 9:451

Katiyar-Agarwal, S., Gao, S., Vivian-smith, A., Jin, H., Katiyar-agarwal, S., Gao, S., Vivian-smith, A., and Jin, H. 2007. A novel class of bacteria-induced small RNAs in Arabidopsis A novel class of bacteria-induced small RNAs in Arabidopsis. Genes Dev. 21:3123–3134

Katiyar-Agarwal, S., Morgan, R., Dahlbeck, D., Borsani, O., Villegas, A., Zhu, J.-K., Staskawicz, B. J., and Jin, H. 2006. A pathogen-inducible endogenous siRNA in plant immunity. Proc. Natl. Acad. Sci. U. S. A. 103:18002–7

Kaufmann, K., Pajoro, A., and Angenent, G. C. 2010. Regulation of transcription in plants: mechanisms controlling developmental switches. Nat. Commun. 11:830–842

Kawahara, Y., Bastide, M. De, Hamilton, J. P., Kanamori, H., Mccombie, W. R., Ouyang, S., Schwartz, D. C., Tanaka, T., Wu, J., Zhou, S., Childs, K. L., Davidson, R. M., Lin, H., Quesada-ocampo, L., Vaillancourt, B., Sakai, H., Lee, S. S., Kim, J., Numa, H., Itoh, T., Buell, C. R., and Matsumoto, T. 2013. Improvement of the Oryza sativa Nipponbare reference genome using next generation sequence and optical map data. Rice. 6:1–10

Keen, N. T., and Kennedy, B. W. 1974. Hydroxyphaseollin and related isoflavanoids in the hypersensitive resistance reaction of soybeans to Pseudomonas glycinea. Physiol. plant Pathol. 4:173–185

Khang, C. H., Berruyer, R., Giraldo, M. C., Kankanala, P., Park, S., Czymmek, K., Kang, S., and Valent, B. 2010. Translocation of Magnaporthe oryzae Effectors into Rice Cells and Their Subsequent Cell-to-Cell Movement. Plant Cell. 22:1388–1403

Khush, G. S. 2005. What it will take to Feed 5 . 0 Billion Rice consumers in 2030. Plant Mol. Biol. 59:1–6

Kim, C. Y., Lee, S. H., Park, H. C., Bae, C. G., Cheong, Y. H., Choi, Y. J., Han, C., Lee, S. Y., Lim, C. O., and Cho, M. J. 2000. Identification of rice blast fungal elicitor-responsive genes by differential display analysis. Mol. Plant. Microbe. Interact. 13:470–474

Komiya, R. 2016. Biogenesis of diverse plant phasiRNAs involves a miRNA-trigger and Dicer-processing. J. Plant Res. DOI 10.100:1–7

Kouassi, N. K., N'Guessan, P., Albar, L., Fauquet, C. M., and Brugidou, C. 2005. Distribution and characterization of rice yellow mottle virus : A Threat to African Farmers. Plant Dis. 89:124–133

Kovach, M. J., Sweeney, M. T., and McCouch, S. R. 2007. New insights into the history of rice domestication. Trends Genet. 23:578–587

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

Lee, H. J., Park, Y. J., Kwak, K. J., Kim, D., Park, J. H., Lim, J. Y., Shin, C., Yang, K.-Y., and Kang, H. 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

Lee, S.-K., Song, M.-Y., Seo, Y.-S., Kim, H.-K., Seho Ko, P.-J. C., Suh, J.-P., Yi, G., Roh, J.-H., Lee, S., An, G., Hahn, T.-R., Wang, G.-L., Ronald, P., and Jeon, J.-

S. 2009. Rice Pi5 -Mediated Resistance to Magnaporthe oryzae Requires the Presence of Two Coiled-Coil–Nucleotide-Binding–Leucine-Rich Repeat Genes. Genetics. 181:1627–1638

Li, F., Pignatta, D., Bendix, C., Brunkard, J. O., Cohn, M. M., Tung, J., and Sun, H. 2011. MicroRNA regulation of plant innate immune receptors. Proc. Natl. Acad. Sci. U. S. A. 109:1790–1795

Li, G., Jain, R., Chern, M., Pham, N. T., Martin, J. A., and Wei, T. 2017a. The Sequences of 1504 Mutants in the Model Rice Variety Kitaake Facilitate Rapid Functional Genomic Studies. Plant Cell. 29:1218–1231

Li, G., and Reinberg, D. 2011. Chromatin higher-order structures and gene regulation. Curr. Opin. Genet. Dev. 21:175–186

Li, X., Xie, X., Li, J., Cui, Y., Hou, Y., Zhai, L., Wang, X., Fu, Y., Liu, R., and Bian, S. 2017b. Conservation and diversification of the miR166 family in soybean and potential roles of newly identified miR166s. BMC Plant Biol. 17:32

Li, Y., Lu, Y.-G., Shi, Y., Wu, L., Xu, Y.-J., Huang, F., Guo, X.-Y., Zhang, Y., Fan, J., Zhao, J.-Q., Zhang, H.-Y., Xu, P.-Z., Zhou, J.-M., Wu, X.-J., Wang, P.-R., and Wang, W.-M. 2014. Multiple rice microRNAs are involved in immunity against the blast fungus Magnaporthe oryzae. Plant Physiol. 164:1077–92

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

Li, Y., Zhao, S.-L., Li, J.-L., Hu, X.-H., Wang, H., Cao, X.-L., Xu, Y.-J., Zhao, Z.-X., Xiao, Z.-Y., Yang, N., Fan, J., Huang, F., and Wang, W.-M. 2017c. Osa-miR169 Negatively Regulates Rice Immunity against the Blast Fungus Magnaporthe oryzae. Front. Plant Sci. 8:1–13

Lin, F., Chen, S., Que, Z., Wang, L., Liu, X., and Pan, Q. 2007a. The Blast Resistance Gene Pi37 Encodes a Nucleotide Binding Site–Leucine-Rich Repeat Protein and Is a Member of a Resistance Gene Cluster on Rice Chromosome 1. Genetics. 177:1871–1880

Lin, R., Zhao, W., Meng, X., Wang, M., and Peng, Y. 2007b. Rice gene OsNAC19 encodes a novel NAC-domain transcription factor and responds to infection by Magnaporthe grisea. Plant Sci. 172:120–130

Ling, K. 1972. Rice virus diseases. Int. Rice Res. Inst. Los Banos, Philipp. :93–105

Liu, B., Chen, Z., Song, X., Liu, C., Cui, X., Zhao, X., Fang, J., Xu, W., Zhang, H., Wang, X., Chu, C., Deng, X., Xue, Y., and Cao, X. 2007a. Oryza sativa dicer-like4 reveals a key role for small interfering RNA silencing in plant development. Plant Cell. 19:2705–18

Liu, B., Li, P., Li, X., Liu, C., Cao, S., Chu, C., and Cao, X. 2005. Loss of Function of OsDCL1 Affects MicroRNA Accumulation and Causes Developmental. 139:296–305

Liu, Q., Feng, Y., and Zhu, Z. 2009. Dicer-like (DCL) proteins in plants. Funct. Integr. Genomics. 9:277–286

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

Liu, X., Lin, F., Wang, L., and Pan, Q. 2007b. The in Silico Map-Based Cloning of Pi36, a Rice Coiled-Coil–Nucleotide-Binding Site–Leucine-Rich Repeat Gene That Confers Race-Specific Resistance to the Blast Fungus. Genetics. 176:2541–2549

López-Sánchez, A., Stassen, J. H., Furci, L., Smith, L. M., and Ton, J. 2016. The role of DNA (de) methylation in immune responsiveness of Arabidopsis. Plant J. 88:361–374

Macho, A. P., and Zipfel, C. 2014. Plant PRRs and the activation of innate immune signaling. Mol. Cell. 54:263–272

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

Martinez-Medina, A., Victor Flors, 2 Martin Heil, Mauch-Mani, B., Pieterse, C. M.., Pozo, M. J., Ton, J., Dam, N. M. van, and Conrath, U. 2016. Recognizing Plant Defense Priming Recognizing Plant. Trends Plant Sci. DOI: 10.10

Mehdy, M. C. 1994. Active Oxygen Species in Plant Defense against Pathogens. Plant phy. 105:467–472

Miao, J., Guo, D., Zhang, J., Huang, Q., Qin, G., Zhang, X., Wan, J., Gu, H., and Qu, L.-J. 2013. Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res. 23:1233–6

Miura, K., Ikeda, M., Matsubara, A., Song, X.-J., Ito, M., Asano, K., Matsuoka, M., Kitano, H., and Ashikari, M. 2010. OsSPL14 promotes panicle branching and higher grain productivity in rice. Nat. Genet. 42:545–549

Miyamoto, K., Nishizawa, Y., Minami, E., Nojiri, H., Yamane, H., and Okada, K. 2015. Overexpression of the bZIP transcription factor OsbZIP79 suppresses the production of diterpenoid phytoalexin in rice cells. J. Plant Physiol. 173:19–27

Morales, F. J., and Niessen, A. I. 1983. Association of spiral filamentous viruslike particles with rice Hoja Blanca. 73:971–974

Nakashima, K., Tran, L. S. P., Van Nguyen, D., Fujita, M., Maruyama, K., Todaka, D., Ito, Y., Hayashi, N., Shinozaki, K., and Yamaguchi-Shinozaki, K. 2007. Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J. 51:617–630

Nalley, L., Tsiboe, F., Durand-morat, A., and Shew, A. 2016. Economic and Environmental Impact of Rice Blast Pathogen (Magnaporthe oryzae) Alleviation in the United States. PLoS One. 11:1–15

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J. D. G. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science (80-.). 312:436–439

Navarro, L., Jay, F., Nomura, K., He, S. Y., and Voinnet, O. 2008. Suppression of the microRNA pathway by bacterial effector proteins. Science (80-.). 321:964–967

Niño-Liu, D. O., Ronald, P. C., and Bogdanove, A. J. 2006. Xanthomonas oryzae pathovars: Model pathogens of a model crop. Mol. Plant Pathol. 7:303–324

Nuruzzaman, M., Manimekalai, R., Sharoni, A. M., Satoh, K., Kondoh, H., Ooka, H., and Kikuchi, S. 2010. Genome-wide analysis of NAC transcription factor family in rice. Gene. 465:30–44

Okada, A., Okada, K., Miyamoto, K., Koga, J., Shibuya, N., Nojiri, H., and Yamane, H. 2009. OsTGAP1, a bZIP Transcription Factor, Coordinately Regulates the Inductive Production of Diterpenoid Phytoalexins in Rice. J. Biol. Chem. 284:26510–26518

Ou-Yang, F., Luo, Q. J., Zhang, Y., Richardson, C. R., Jiang, Y., and Rock, C. D. 2013. Transposable element-associated microRNA hairpins produce 21-nt sRNAs integrated into typical microRNA pathways in rice. Funct. Integr. Genomics. 13:207–216

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

425:257–263

Palm, M. E. 1995. Plectosporium, a new genus for Fusarium tabacinum, the anamorph of Plectosphaerella cucumerina. Mycologia. 87:397–406

Park, Y. J., Lee, H. J., Kwak, K. J., Lee, K., Hong, S. W., and Kang, H. 2014. MicroRNA400-guided Cleavage of Pentatricopeptide Repeat Protein mRNAs Renders Arabidopsis thaliana More Susceptible to Pathogenic Bacteria and Fungi. Plant Cell Physiol. 55:1660–8

Pastor, V., Luna, E., Ton, J., Cerezo, M., García-agustín, P., and Flors, V. 2013. Fine Tuning of Reactive Oxygen Species Homeostasis Regulates Primed Immune Responses in Arabidopsis. MPMI. 26:1334–1344

Pavet, V., Quintero, C., Cecchini, N. M., Rosa, A. L., and Alvarez, M. E. 2006. Arabidopsis Displays Centromeric DNA Hypomethylation and Cytological Alterations of Heterochromatin Upon Attack by Pseudomonas syringae. MPMI. 19:577–587

Paxton, J. 1980. A New Working Definition of the Term “Phytoalexin.” Plan Dis. 64:734

Pinweha, N., Asvarak, T., Viboonjun, U., and Narangajavana, J. 2015. Involvement of miR160/miR393 and their targets in cassava responses to anthracnose disease. J. Plant Physiol. 174:26–35

Qiu, Y., and Yu, D. 2009. Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in Arabidopsis. Environ. Exp. Bot. 65:35–47

Qu, S., Liu, G., Zhou, B., Bellizzi, M., Zeng, L., Dai, L., Han, B., and Wang, G. 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. Genetics. 172:1901–1914

Rajagopalan, R., Vaucheret, H., Trejo, J., and 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., and 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

Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B., and Bartel, D. P. 2002. Prediction of plant microRNA targets. Cell. 110:513–520

Rogers, K., and Chen, X. 2013a. Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell. 25:2383–99

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

Sanchez, P. L., Wing, R. A., and Brar, D. S. 2013. The Wild Relative of Rice. Genomes and Genomics.9-25

Sato, N., Kitazawa, K., and Tomiyama, K. 1971. The role of rishitin in localizing the invading hyphae of Phytophthora infestans in infection sites at the cut surfaces of potato tubers. Physiol. Plant Pathol. 1:289–295

Sesma, A., and Osbourn, A. E. 2004. The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. Nature. 431:582–586

Shang, J., Tao, Y., Chen, X., Zou, Y., Lei, C., and Wang, J. 2009. Identification of a New Rice Blast Resistance Gene, Pid3, by Genomewide Comparison of Paired Nucleotide-Binding Site–Leucine-Rich Repeat Genes and Their Pseudogene Alleles Between the Two Sequenced Rice Genomes. Genetics. 182:1303–1311

Shimura, K., Okada, A., Okada, K., Jikumaru, Y., Ko, K. W., Toyomasu, T., Sassa, T., Hasegawa, M., Kodama, O., Shibuya, N., Koga, J., Nojiri, H., and Yamane, H. 2007. Identification of a biosynthetic gene cluster in rice for momilactones. J. Biol. Chem. 282:34013–34018

Shivaprasad, P. V, Chen, H., Patel, K., Bond, D. M., Santos, B. A. C. M., and Baulcombe, D. C. 2012. A MicroRNA Superfamily Regulates Nucleotide Binding Site – Leucine-Rich Repeats and Other mRNAs. Plant Cell. 24:859–874

Song, X., Li, P., Zhai, J., Zhou, M., Ma, L., Liu, B., Jeong, D.-H., Nakano, M., Cao, S., Liu, C., Chu, C., Wang, X.-J., Green, P. J., Meyers, B. C., and Cao, X. 2012. Roles of DCL4 and DCL3b in rice phased small RNA biogenesis. Plant J. 69:462–474

Soto-Suarez, M., Baldrich, P., Weigel, D., Rubio-Somoza, I., and San Segundo, B. 2017. The Arabidopsis miR396 mediates pathogen-associated molecular pattern-triggered immune responses against fungal pathogens. Sci. Rep. 7:1–14

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

Stergiopoulos, I., van den Burg, H. A., Okmen, B., Beenen, H. G., van Liere, S., Kema, G. H. J., and de Wit, P. J. G. M. 2010. Tomato Cf resistance proteins mediate recognition of cognate homologous effectors from fungi pathogenic on dicots and monocots. Proc. Natl. Acad. Sci. 107:7610–7615

Sun, L., Zhang, H., Li, D., Huang, L., Hong, Y., Ding, X. S., Nelson, R. S., Zhou, X., and Song, F. 2013. Functions of rice NAC transcriptional factors, ONAC122 and ONAC131, in defense responses against Magnaporthe grisea. Plant Mol. Biol. 81:41–56

Sunkar, R., Li, Y. F., and Jagadeeswaran, G. 2012. Functions of microRNAs in plant stress responses. Trends Plant Sci. 17:196–203

Sweeney, M., and McCouch, S. 2007. The Complex History of the Domestication of Rice. Ann. Bot. 100:951–957

Swings, J., Van den Mooter, M., Vauterin, L., Hoste, B., Gillis, M., Mew, T., and Kersters, K. 1990. Reclassification of the Causal Agents of Bacterial Blight (Xanthomonas campestris pv. oryzae) and Bacterial Leaf Streak (Xanthomonas campestris pv. oryzicola. Int. J. Syst. BACTERIOL. 40:309–311

Tamaoki, D., Seo, S., Yamada, S., Kano, A., Miyamoto, A., Shishido, H., Miyoshi, S., Taniguchi, S., Akimitsu, K., and Gomi, K. 2013. Jasmonic acid and salicylic acid activate a common defense system in rice. Plant signal Behav. 8:8–10

Tanweer, F. A., Rafii, M. Y., Sijam, K., Rahim, H. A., Ahmed, F., and Latif, M. A. 2015. Current advance methods for the identification of blast resistance genes in rice. C. R. Biol. 338:321–34

The 1001 genomes. 1001genomes.org

The 3000 rice genomes project. 2014. The 3,000 rice genomes project. Gigascience. 3:7

The Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature. 408:796–815

Ton, J., and Mauch-Mani, B. M. 2004. b -amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J. 38:119–130

Tsuji, J., Jackson, E. P., Gage, D. A., Somerville, S. C., State, M., Energy, U., and Nuclear, M. T. R. 1992. Phytoalexin Accumulation in Arabidopsis thaliana during the Hypersensitive Reaction to Pseudomonas syringae pv syringae.

Plant Physiol. 98:1304–1309

Van der Linden, L., Bredenkamp, J., Naidoo, S., Fouché-Weich, J., Denby, K. J., Genin, S., Marco, Y., and Berger, D. K. 2013. Gene-for-gene tolerance to bacterial wilt in Arabidopsis. Mol. Plant. Microbe. Interact. 26:398–406

Van Loon, L. C. 1999. Occurrence and Properties of Plant Pathogenesis-Related Proteins. CRC Press.1–19

VanLoon, L. C., Rep, M., and Pieterse, C. M. J. 2006. Significance of Inducible Defense-related Proteins in Infected Plants. Annu. Rev. Phytopathol. 44:135–62

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

Voinnet, O. 2009. Origin, Biogenesis, and Activity of Plant MicroRNAs. Cell. 136:669–687

Volpe, T. 2002. Regulation oh heterochromatic silencing and histone H3 Lysine-9 by RNAi. Science (80-.). 297:1833–1837

Wang, H. H., Peng, X. X., Tang, X. K., Zhou, P. L., Hu, Y. J., Deng, X. B., and He, Y. 2011a. Isolation and Expression Patterns of Rice WRKY82 Transcription Factor Gene Responsive to Both Biotic and Abiotic Stresses. Agric. Sci. China. 10:893–901

Wang, M., Yu, Y., Haberer, G., Marri, P. R., Fan, C., Goicoechea, J. L., Zuccolo, A., Song, X., Kudrna, D., Ammiraju, J. S. S., Cossu, R. M., Maldonado, C., Chen, J., Lee, S., Sisneros, N., de Baynast, K., Golser, W., Wissotski, M., Kim, W., Sanchez, P., Ndjiondjop, M.-N., Sanni, K., Long, M., Carney, J., Panaud, O., Wicker, T., Machado, C. A., Chen, M., Mayer, K. F. X., Rounsley, S., and Wing, R. A. 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., Zeng, R., Zhu, H., Dong, G., Qian, Q., Zhang, G., and Fu, X. 2012. Control of grain size, shape and quality by OsSPL16 in rice. Nat. Genet. 44:950–4

Wang, X.-B., Jovel, J., Udomporn, P., Wang, Y., Wu, Q., Li, W.-X., Gasciolli, V., Vaucheret, H., and Ding, S.-W. 2011b. The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative argonautes in Arabidopsis thaliana. Plant Cell. 23:1625–1638

Wang, Z., Yano, M., and Yamanouchi, U. 1999. The Pib gene for rice blast resistance belongs to the nucleotide binding and leucine-rich repeat class of plant disease resistance genes. Plant J. 19:55–64

Wilson, R. A., and Talbot, N. J. 2009. Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. Nat. Rev. 7:185–195

Wu, J., Yang, Z., Wang, Y., Zheng, L., Ye, R., Ji, Y., Zhang, X., Cao, X., Xie, L., Wu, Z., Qi, Y., and Li, Y. 2015. Viral-inducible Argonaute18 confers broad-spectrum virus resistance in rice by sequestering a host microRNA. Elife. 4:1–19

Wulff, E. G., Sørensen, J. L., Lübeck, M., Nielsen, K. F., Thrane, U., and Torp, J. 2010. Fusarium spp. associated with rice Bakanae: Ecology, genetic diversity, pathogenicity and toxigenicity. Environ. Microbiol. 12:649–657

Xia, K., Wang, R., Ou, X., Fang, Z., Tian, C., Duan, J., Wang, Y., and Zhang, M. 2012. OsTIR1 and OsAFB2 downregulation via OsmiR393 overexpression leads to more tillers, early flowering and less tolerance to salt and drought in rice. PLoS One. 7:1–10

Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E., and Carrington, J. C. 2004. Genetic and functional diversification of small RNA pathways in plants. PLoS Biol. 2:642–652

Xing, H.-L., Dong, L., Wang, Z.-P., Zhang, H.-Y., Han, C.-Y., Liu, B., Wang, X.-C., and Chen, Q.-J. 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol. 14:327

Xu, M., Wilderman, P. R., Morrone, D., Xu, J., Roy, A., Margis-pinheiro, M., Upadhyaya, N. M., Coates, R. M., and Peters, R. J. 2007. Functional characterization of the rice kaurene synthase-like gene family. Phytochemistry. 68:312–326

Yamamura, C., Mizutani, E., Okada, K., Nakagawa, H., Fukushima, S., and Tanaka, A. 2015. Diterpenoid phytoalexin factor, a bHLH transcription factor, plays a central role in the biosynthesis of diterpenoid phytoalexins in rice. plant J. 84:1100–1113

Yamane, H. 2013. Biosynthesis of Phytoalexins and Regulatory Mechanisms of It in Rice. Biosci. Biotechnol. Biochem. 77:1141–1148

Yan, L. I., Bai-yuan, Y. A. N., Yun-liang, P. E. N. G., and Yu-xiang, Z. E. N. G. 2017. Molecular screening of blast resistance genes in rice germoplasms resistant to Magnaporthe oryzae. Rice Sci. 24:41–47

Yellareddygari, S., Reddy, M., Kloepper, J., Lawrence, K., and Fadamiro, H. 2014. Rice Sheath Blight: A Review of Disease and Pathogen Management Approaches. J. Plant Pathol. Microbiol. 5:1–4

Yoshikawa, M., Yamauchi, K., and Masago, H. 1978. Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infected with Phytophthora megasperma var. sojae. Physiol. Plant Pathol. 12:73–82

Yu, J., Hu, S., Wang, J., Wong, G. K., Li, S., Liu, B., Deng, Y., Dai, L., Zhou, Y., Zhang, X., Cao, M., Liu, J., Sun, J., Tang, J., Chen, Y., Huang, X., Lin, W., Ye, C., Tong, W., Cong, L., Geng, J., Han, Y., Li, L., Li, W., Hu, G., Huang, X., Li, W., Li, J., Liu, Z., Li, L., Liu, J., Qi, Q., Liu, J., Li, L., Li, T., Wang, X., Lu, H., Wu, T., Zhu, M., Ni, P., Han, H., Dong, W., Ren, X., Feng, X., Cui, P., Li, X., Wang, H., Xu, X., Zhai, W., Xu, Z., Zhang, J., He, S., Zhang, J., Xu, J., Zhang, K., Zheng, X., Dong, J., Zeng, W., Tao, L., Ye, J., Tan, J., Ren, X., Chen, X., He, J., Liu, D., Tian, W., Tian, C., Xia, H., Bao, Q., Li, G., Gao, H., Cao, T., Wang, J., Zhao, W., Li, P., Chen, W., Wang, X., Zhang, Y., Hu, J., Wang, J., Liu, S., Yang, J., Zhang, G., Xiong, Y., Li, Z., Mao, L., Zhou, C., Zhu, Z., Chen, R., Hao, B., Zheng, W., Chen, S., and Guo, W. 2002. A Draft Sequence of the Rice Genome (Oryza sativa L. ssp. indica). Science (80-.). 296:79–93

Zeigler, R., Leong, S., and Teng, P. 1994. Rice blast disease. Commonw. Agric. Bur. Wallingford, UK. :626

Zhai, J., Jeong, D. H., de Paoli, E., Park, S., Rosen, B. D., Li, Y., González, A. J., Yan, Z., Kitto, S. L., Grusak, M. A., Jackson, S. A., Stacey, G., Cook, D. R., Green, P. J., Sherrier, D. J., and Meyers, B. C. 2011. MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. Genes Dev. 25:2540–2553

Zhang, D., Liu, M., Tang, M., Dong, B., Wu, D., Zhang, Z., and Zhou, B. 2015. Repression of microRNA biogenesis by silencing of OsDCL1 activates the basal resistance to Magnaporthe oryzae in rice. Plant Sci. 237:24–32

Zhang, J., Peng, Y., and Guo, Z. 2008. Constitutive expression of pathogen-inducible OsWRKY31 enhances disease resistance and affects root growth and auxin response in transgenic rice plants. Cell Res. 18:508–521

Zhang, W., Gao, S., Zhou, X., Chellappan, P., Chen, Z., Zhou, X., Zhang, X., Fromuth, N., Coutino, G., Coffey, M., and Jin, H. 2011a. Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. Plant Mol. Biol. 75:93–105

Zhang, X., Zhao, H., Gao, S., Wang, W.-C., Katiyar-Agarwal, S., Huang, H.-D., Raikhel, N., and Jin, H. 2011b. Arabidopsis Argonaute 2 regulates innate immunity via miRNA393*-mediated silencing of a Golgi-localized SNARE gene MEMB12. Mol. Cell. 42:356–366

Zhang, Y., Yu, Y., Wang, C., Li, Z., Liu, Q., Xu, J., Liao, J., Wang, X., Qu, L., Chen, F., Xin, P., Yan, C., Chu, J., Li, H., and Chen, Y. 2013. Overexpression of microRNA OsmiR397 improves rice yield by increasing grain size and promoting panicle branching. Nat. Biotechnol. 31:848–852

Zhou, B., Qu, S., Liu, G., Dolan, M., Sakai, H., Lu, G., Bellizzi, M., and Wang, G. 2006. The Eight Amino-Acid Differences Within Three Leucine-Rich Repeats Between Pi2 and Piz-t Resistance Proteins Determine the Resistance Specificity to Magnaporthe grisea. Mol. plant microbe Interact. 19:1216–1228

Zhu, Q. H., Fan, L., Liu, Y., Xu, H., Llewellyn, D., and Wilson, I. 2013. miR482 regulation of NBS-LRR defense genes during fungal pathogen infection in cotton. PLoS One. 8

Zilberman, D., Coleman-Derr, D., Ballinger, T., and Henikoff, S. 2008. Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature. 456:125–9

Annex

Polycistronic miR1861b-1861c and miR5534a-5534b from rice

In addition to miR166k-166h, during the course of this work we investigated two additional polycistronic miRNA precursors encoding homologous miRNAs. They were: miR1861b-1861c and miR5534a-5534b. Rice mutant lines for these polycistronic miRNAs were identified in the TRIM (Taiwan Rice Insertional Mutants) collection, two mutant lines for *MIR1861b-1861c* (M0028810, M0113622), and one mutant line for *MIR5534a-5534b* (M0007961). These lines were genotyped and further characterized.

The polycistronic miR1861b-1861c is a positive regulator of the rice immunity against *M. oryzae*

Characterization of rice miR1861b-1861c activation mutants

Two candidate lines potentially representing activation lines for miR1861b-1861c were obtained (TRIM collection; M0028810, M0113622). These lines were generated in the TN67 genotype (*japonica* cultivar). They were PCR-genotyped using specific primers located in the *MIR1861b-1861c* locus (P1, P2), left border of the T-DNA (P3) and the genomic region near the T-DNA insertion site (P5). As result, we identified hemizygous (He) and azygous (Az) lines for the T-DNA (**Fig. 1A**). In addition, miR1861b-1861c precursor transcripts accumulated at a higher level in the rice mutant lines (*miR1861-Ac#1, miR1861-Ac#2*), thus, confirming that these lines are activation mutants for *MIR1861b-1861c* (**Fig. 1B**, left panel). When examining the expression of other genes located near the T-DNA insertion site, namely *neutral invertase* gene (in the *miR1861-Ac#1* and *miR1861-Ac#2* mutants) and *exosome complex exonuclease* (in the *miR1861-Ac#1* mutant) we found that none of these genes is affected by the T-DNA insertion (**Fig. 1B**, middle and right panels).

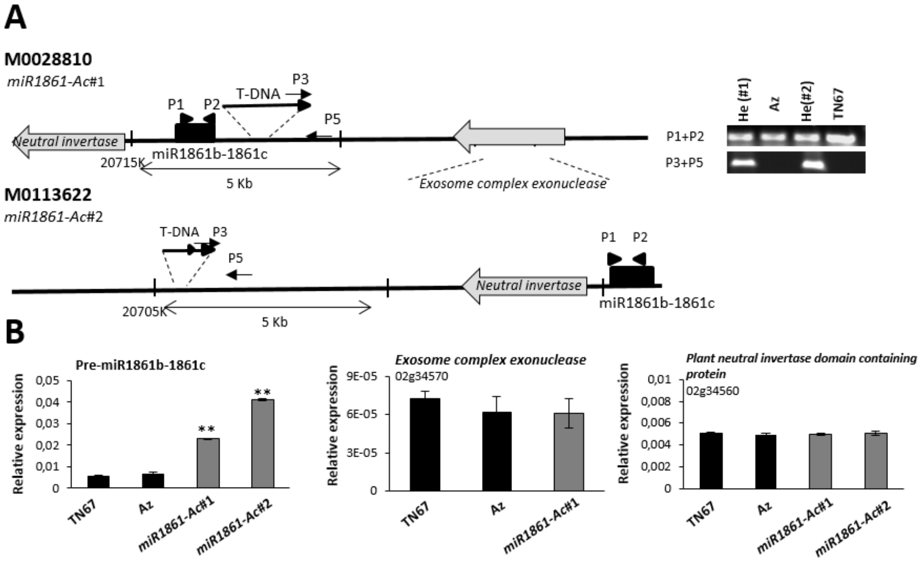


Figure 1. Characterization of rice miR1861b-1861c mutants. A. Activation lines for miR1861b-1861c (*miR1861-Ac#1, miR1861-Ac#2*) were genotyped by PCR using miRNA-specific primers (P1 and P2, arrowheads) and T-DNA flanking primers (P5, arrows). In addition, a primer designed in the LB region of T-DNA (P3) was also used for genotyping. **B.** The accumulation of miR1861b-1861c precursor transcripts in TN67 wild-type, segregated azygous and activation lines was determined by RT-qPCR (left panel). The accumulation of transcripts for genes located in the vicinity of the T-DNA insertion site is also shown (middle and right panels). Histograms show the mean ± SD (**, p-value ≤0.01; ANOVA test).

Resistance to infection by the rice blast fungus in rice activation-tagged mutants for *MIR1861b-1861c*

Wild-type TN67 plants, azygous and polycistronic miR1861b-1861c activation lines (hereinafter *miR1861-Ac#1*, *miR1861-Ac#2*) were challenged with *M. oryzae*. Three-week old rice plants were inoculated with fungal spores (5 x 10^5 spores/mL) and disease symptoms were evaluated with time. The *miR1861-Ac* lines exhibited reduced blast symptoms (**Fig.2**, left panel). Blast resistance was confirmed by fungal biomass quantification and lesion area measurements (**Fig. 2**, right panel).

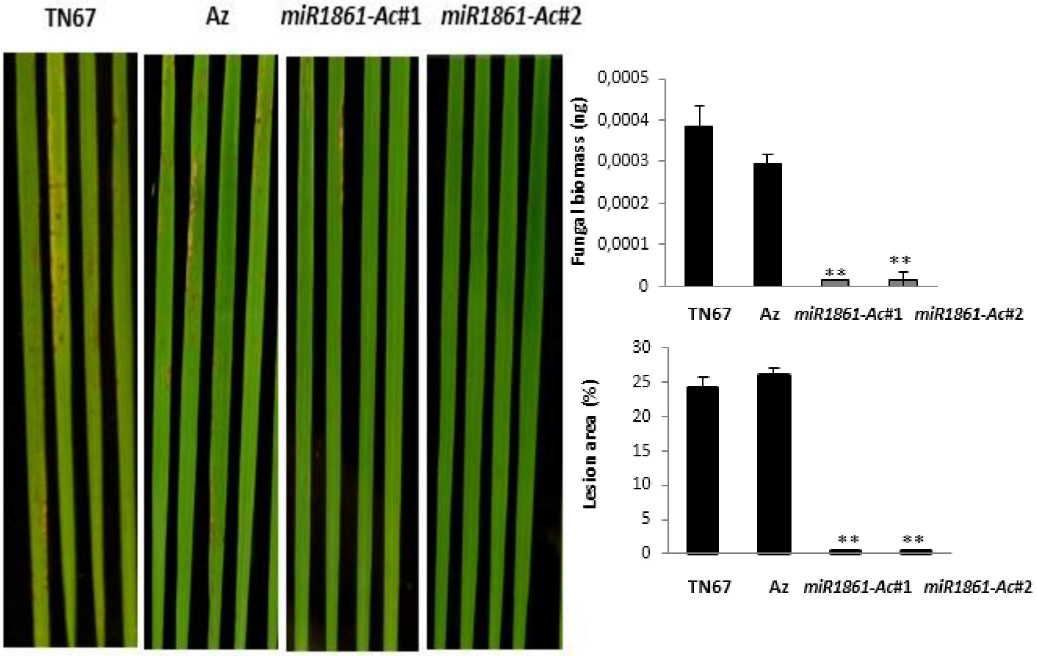


Figure 2. **Enhanced resistance of miR1861b-1861c activation mutants to infection by the rice blast fungus *M. oryzae*.** Phenotype of wild-type (TN67), azygous (Az) and miR1861b-1861c (*miR1861-Ac*#1, M0028810; *miR1861-Ac*#2, M0113622) activation plants at 7 days post-inoculation with *M. oryzae* spores (5 x 10^5 spores/mL). Lesion area was determined by the image software Asses 2.0, and *M. oryzae* biomass was measured by qPCR on genomic DNA. Results from three biological replicates and at least 24 plants per genotype are shown. Histograms show the mean ± SD (**, p-value ≤ 0.01, ANOVA test).

Next, we examined the expression of defense-related genes in mutant and wild-type plants during infection by *M. oryzae*. As it is shown in **Figure 3**, *PBZ1* and *PR1a* expression was induced at a higher level in *miR1861-Ac* plants than in wild-type plants. Differences were more evident at 72 hours post-inoculation.

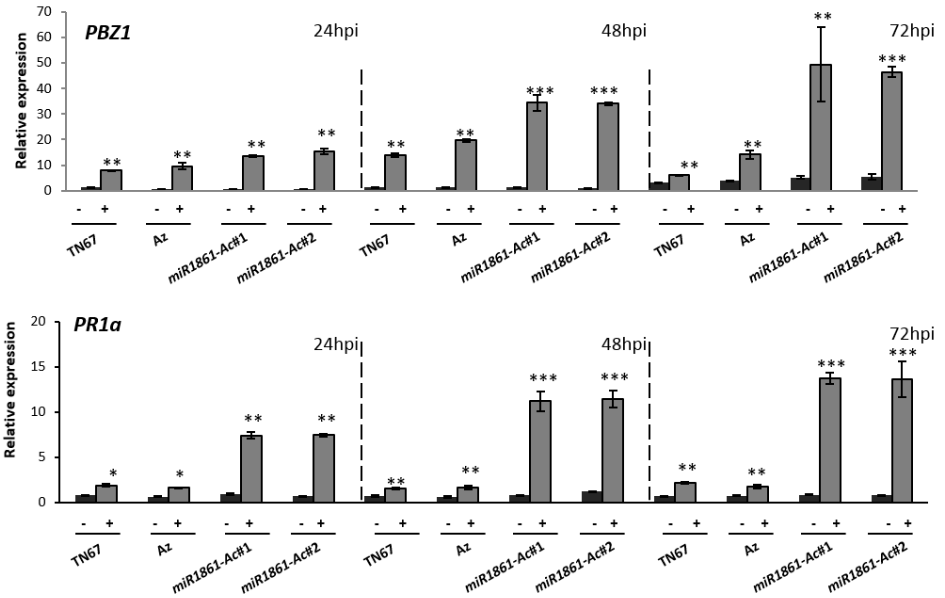


Figure 3. Expression of the defense-related genes *PBZ1* and *PR1a* in wild-type (TN67), azygous and miR1861b-1861c activation lines at the indicated times after infection with *M. oryzae* spores (5 x 10^5 spores/mL) (grey bars). As control, plants were mock-inoculated (black bars). Transcript accumulation was measured by RT-qPCR using the *cyclophilin* gene as the endogenous reference gene. Results from three biological replicates and at least 24 plants per genotype and condition. Histograms show the mean ± SD (***, p-value ≤ 0.001; **, p-value ≤ 0.01; *, p-value ≤ 0.05, determined by ANOVA).

We also investigated whether the accumulation of miR1861b-1861c precursor and mature sequences is regulated during pathogen infection. RT-qPCR analysis revealed an increase in the accumulation of miR1861b-1861c transcripts in wild-type plants at all the time points of infection here examined (**Fig. 4A**, left panel). The observed increase in precursor transcripts is accompanied by an increase in the accumulation of mature miR1861 sequences encoded by the miR1861b-1861c precursor (**Fig. 4A,** middle and right panels). As there are no monocistronic loci for either miR1861b or miR1861c in the rice genome, miR1861b and miR1861c species are expected to be produced by miR1861b-1861c processing. To note, the accumulation of miR1861b-miR1861c precursor transcripts, and mature miR1861 species encoded by this precursor, increased in response to treatment with elicitors obtained from *M. oryzae* (**Fig. 4B**). This observation supports that the polycistronic miR1861b-1861c functions in PTI against *M. oryzae.*

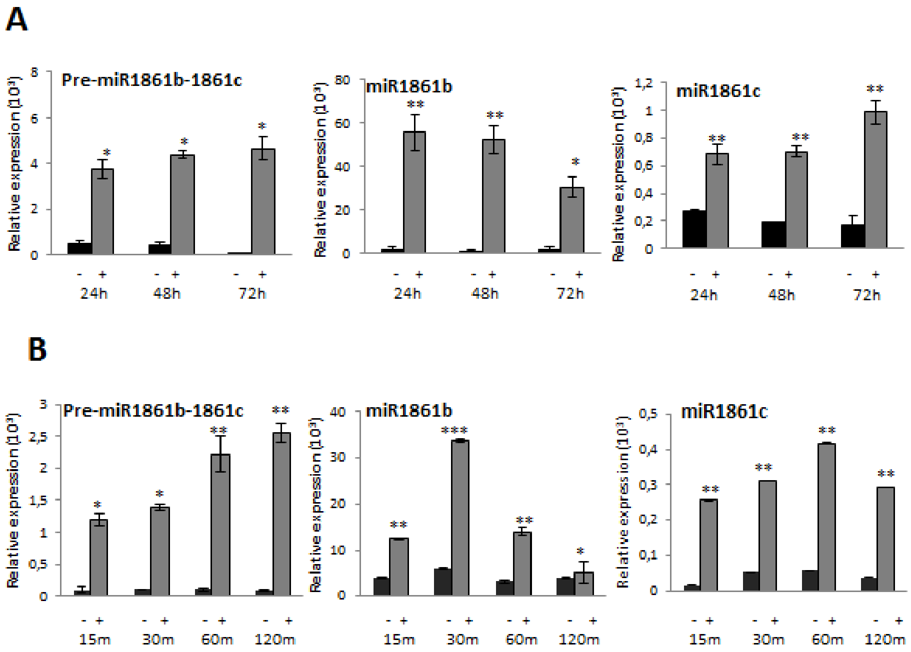


Figure 4. Accumulation of miR1861b-1861c transcripts in response to *M. oryzae* infection and treatment with fungal elicitors in wild-type plants. A. Expression at different times after inoculation with *M. oryzae* spores (5 x 10^5 spores/mL). **B.** Expression in response to treatment with *M. oryzae* elicitors (300 µg/mL). The accumulation of miR1861b-1861c precursor and mature miR1861 sequences was determined by RT-qPCR using *cyclophilin* as the reference gene. Mock-inoculated plants are represented by black bars, whereas *M. oryzae*-inoculated (A) or elicitor-treated (B) plants are represented by grey bars. Three independent experiments were carried out. Histograms show the mean ± SD (***, p-value ≤ 0.001; **, p-value ≤ 0.01; *, p-value ≤ 0.05, ANOVA).

Expression of miR1861 target genes during infection with the rice blast fungus *M. oryzae*

Knowing that *M. oryzae* infection increases miR1861b-1861c accumulation in rice leaves, it was of interest to investigate the expression of miR1861 target genes in the same tissues. For miR1861c, a *glyoxalase* gene encoding an enzyme of the glioxylate cycle has been previously identified by degradome analysis (Baldrich et al. 2015). In this work, two additional target genes were predicted for miR1861 family members encoded by the polycistronic miR1861b-1861c precursor using psRNATarget software (http://plantgrn.noble.org/psRNATarget/). They were: *ATP binding protein* (as a putative target of miR1861b), and *plus-3 domain containing protein* genes (as a putative target of miR1861c) (**Fig. 5A**). Consistent with the observed increase in miR1861b-1861c precursor transcripts during *M. oryzae* infection (see **Fig. 4**), transcripts for *glioxylase* and the two predicted target genes for miR1861b-1861c decreased at the same time of infection (72hpi with *M. oryzae*) (**Fig. 5B**). Treatment with *M. oryzae* elicitors also results in a remarkable reduction on the accumulation of *glioxylase, ATP binding protein and Plus-3-domain containing protein transcripts* (**Fig. 5C**).

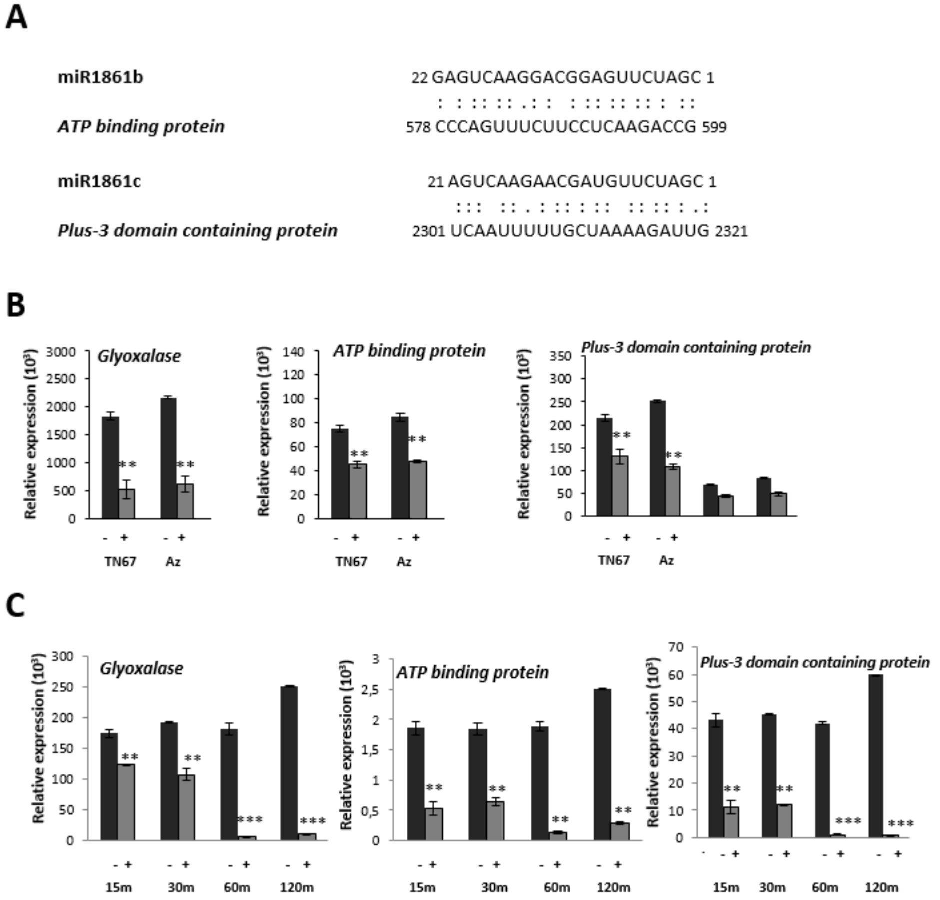


Figure 5. Accumulation of transcripts for known (*glyoxilase*) and predicted (*ATP binding protein* and *plus-3 domain containing protein*) target genes for miR1861b-1861c in wild-type plants. A. Alignement of miR1861b and miR1861c with their predicted target genes. B. Expression of the indicated genes was determined by RT-PCR in control (black bars) and *M. oryzae*-infected (72 hpi; grey bars) in wild-type TN67 and azygous plants. C. Expression in wild-type plants that have been treated, or not, with *M. oryzae* elicitors. Results from three biological replicates and at least 24 plants per condition are shown. Histograms show the mean ± SD (***, p-value ≤ 0.001 **, p-value ≤ 0.01; *, p-value ≤ 0.05, ANOVA test).

Collectively, results obtained on the analysis of miR1861b-1861c mutant plants suggest that the polycistronic miR1861b-1861c is a positive regulator of PTI in rice. Although accumulation of miR1861b-miR1861c transcripts inversely correlated with that of target transcripts, including transcripts for the two predicted target genes, further investigation is needed to determine whether the *ATP binding protein* and the *plus-3 domain containing protein* are real targets for miR1861b-1861c.

The polycistronic miR5534a-5534b is a positive regulator of the rice immunity against *M. oryzae*

Characterization of rice miR5534a-5534b activation mutants

Our search on the TRIM collection identified a candidate activation mutant for the *MIR5534a-5534b* locus (M0007961). Plants were PCR-genotyped using specific primers located in the *MIR5534a-5534b* locus (P1, P2), left border of the T-DNA (P3) and flanking region of the T-DNA insertion site (P5). Homozygous (Ho) plants for the T-DNA were identified for this mutant (**Fig. 6A**). Sequencing of the RT-PCR products confirmed that the miR5534a-5534b precursor is transcribed as a single transcriptional unit that comprises miR5534a and miR5534b sequences. The fact that this mutant is an activation mutant for *MIR5534a-5534b* was also confirmed by determining the accumulation of precursor transcripts (**Fig. 6B**, left panel). We will refer to this mutant as *miR5534-Ac*. The T-DNA flanking gene, *Embryo-sac basal-endosperm layer embryo-surrounding-region* (*ZmEBE-1*), located downstream of the *MIR5534a-5534b* locus, was found not to be affected by the T-DNA insertion (**Fig. 6B**, right panel).

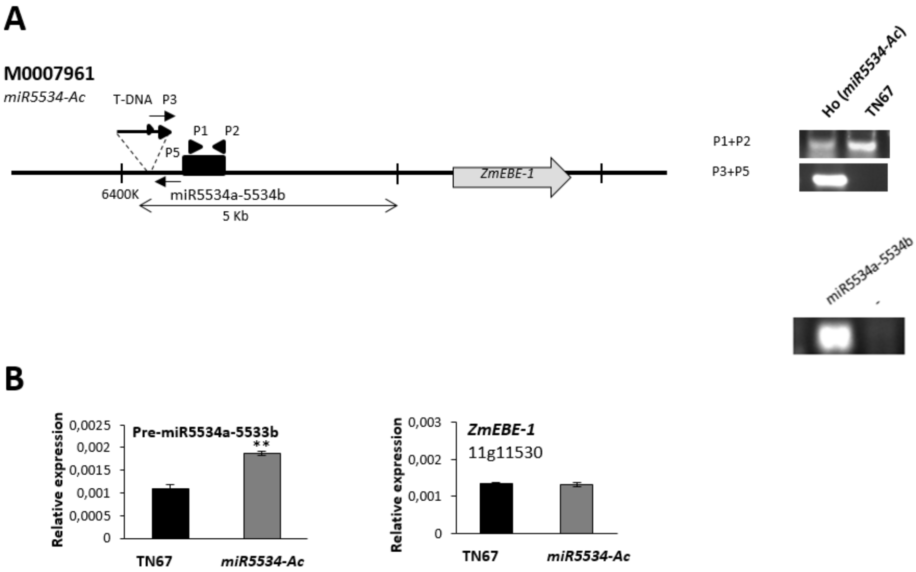


Figure 6. Characterization of the rice miR5534a-5534b mutant. A. PCR-genoyting of the miR5534a-5534b mutant using miRNA-specific primers (P1 and P2, black arrowheads) and T-DNA flanking primers (P5, arrows). A primer designed in the LB region of T-DNA (P3) was also used for genotyping. B. Accumulation of miR5534a-5534b precursor transcripts and *ZmEBE-1* transcripts was determined by RT-qPCR. Histograms show the mean ± SD (**, p-value ≤0.01; ANOVA test).

Resistance to infection by the rice blast fungus in rice activation-tagged mutants for *MIR5534a-5534b*

Wild-type and miR5534a-5534b mutant plants were challenged with *M. oryzae*. Three-week old rice plants were inoculated with fungal spores (5 x 10^5 spores/mL) and disease symptoms were followed with time. As it is shown in **Fig. 7** (left panel)**,** the *miR5534-Ac* line exhibited resistance to infection by the rice blast fungus *M. oryzae*. Fungal biomass quantification and lesion area measurements confirmed blast disease resistance in the miR5534a-5534b mutant plants (**Fig. 7**, right panel).

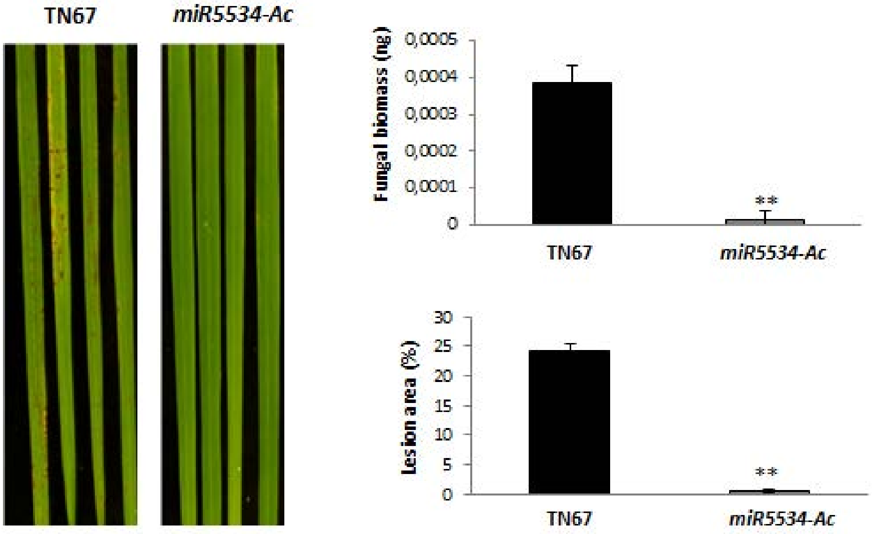


Figure 7. Characterization of the miR5534a-5534b activation mutant with *M. oryzae* infection. Phenotype of wild-type (TN67) and miR5534a-5534b (*miR5534-Ac*, M0007961) activation plants at 7 days post-inoculation with *M. oryzae* spores (5 x 10^5 spores/mL). Lesion area was determined by the image software Asses 2.0, and *M. oryzae* biomass was measured by qPCR in genomic DNA. Results from three biological replicates and at least 24 plants per genotype are shown. Histograms show the mean ± SD (**, p-value ≤ 0.01, determined by ANOVA).

As for the other polycistronic miRNA under study, we examined the expression of defense-related genes *PBZ1* and *PR1a*. Upon challenge with *M. oryzae*, *PBZ1* and *PR1a* were more highly up-regulated in the mutant plants than in wild-type plants (**Fig. 8**). This observation is consistent with the resistance phenotype that is observed in the *miR5534-Ac* mutant plants.

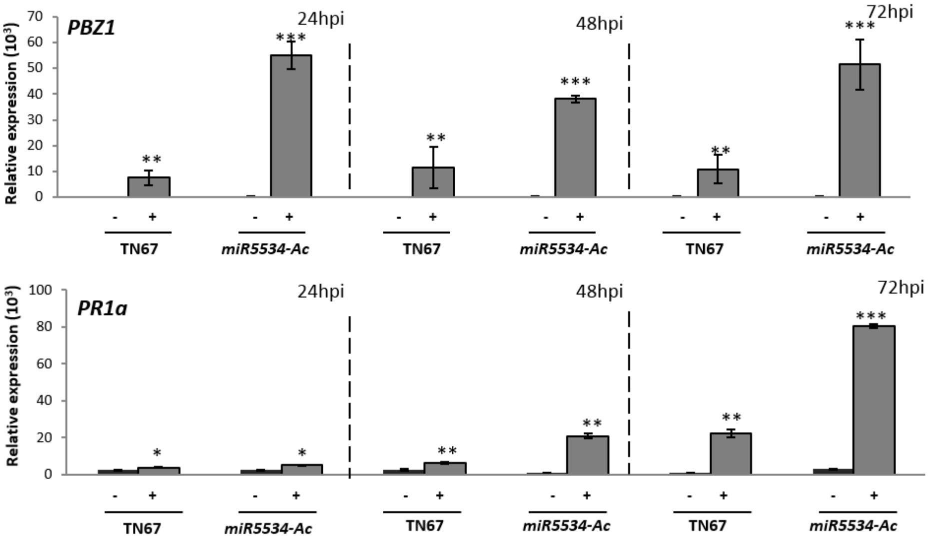


Figure 8. Expression of the defense-related genes *PBZ1* and *PR1a* in wild-type (TN67) and miR5534a-5534b activation line at the indicated times after infection with *M. oryzae* spores (5 x 10^5 spores/mL) (grey bars). As control, plants were mock-inoculated (black bars). Transcript accumulation was measured by RT-qPCR using the *cyclophilin* gene as the endogenous reference gene. Results from three biological replicates and at least 24 plants per genotype and condition. Histograms show the mean ± SD (***, p-value ≤ 0.001; **, p-value ≤ 0.01; *, p-value ≤ 0.05, determined by ANOVA).

RT-qPCR was used to analyze the transcript accumulation profile of *MIR5534a-5534b* in wild-type plants during *M. oryzae* infection and treatment with *M. oryzae* elicitors. There was a general increase in the accumulation of miR5534a-5534b precursor transcripts, as well as mature miR5534a and miR5534b sequences in response to *M. oryzae* infection (**Fig. 9A**). A similar trend was observed after treatment with *M. oryzae* elicitors (**Fig. 9B**). Again, as the rice genome does not contain monocistronic miR5534a or miR5534b loci, accumulation of mature miR5534 species would reflect miR5534a-miR5534b precursor processing events. The observation that the accumulation of miR5534a-5534b transcripts respond to treatment with fungal elicitors (a response to fungal perception) supports that the polycistronic miR1861b-1861c functions in PTI against *M. oryzae.*

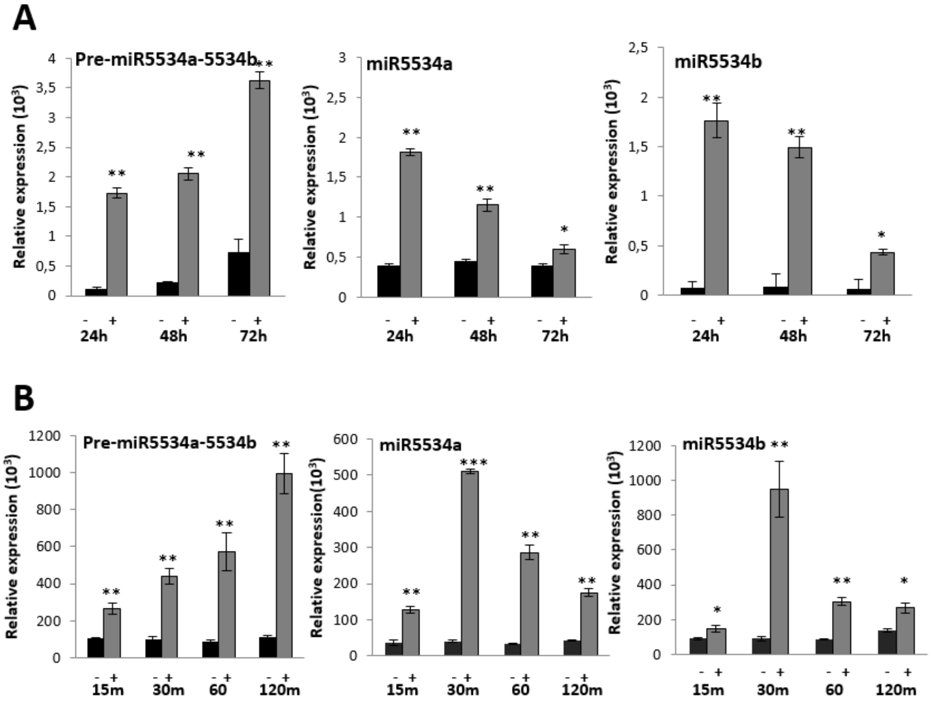


Figure 9. Accumulation of *MIR5534a-5534b* transcripts in wild-type plants in response to *M. oryzae* infection. A. Expression after inoculation with *M. oryzae* spores (5 x 10^5 spores/mL). B. Expression in response to treatment with *M. oryzae* elicitors (300 µg/mL). The accumulation of miR5534a-5534b precursor and mature miR5534 sequences was determined by RT-qPCR. Plants were inoculated with *M. oryzae* spores or with a suspension of fungal elicitors (grey bars), or mock-inoculated plants (black bars). Three independent experiments were carried out. Histograms show the mean ± SD (***, p-value ≤ 0.001; **, p-value ≤ 0.01; *, p-value ≤ 0.05, ANOVA test).

Expression of candidate target genes for miR5534 during infection with the rice blast fungus *M. oryzae*

No target gene has been described in literature for the rice miR5534a or miR534b yet. Based on target prediction using the psRNATarget software, miR5534a and miR5534b were predicted to target a *DUF26 kinase* (*Domain of Unknown Function 26 kinase*). Additionally, a *α-glucosidase* gene was also predicted as a target gene for miR5534b (**Fig 10A**). DUF26 kinases are cystein-rich receptor-like kinases that mediate plant immunity responses to pathogen infection. The DUF26 kinases group comprises nearly 40 members and it is thought to function directly as a sensor of ROS (Reactive Oxigen Species) in the apoplast (Wrzaczek et al. 2010; Rayapuram et al. 2012). Consistent with the observed increase in miR5534a-5534b precursor transcript levels during *M. oryzae* infection and elicitor treatment (see **Fig. 9**), *DUF26 kinase* transcript levels decreased in *M. oryzae*-infected wild-type plants (72hpi) (**Fig. 10B**, left panel). A slight, but significant decrease in the accumulation of *α-glucosidase* transcripts was also observed in *M. oryzae*-infected plants (**Fig. 10B**, right panel). Finally, treatment with *M. oryzae* elicitors also results in a reduction of the accumulation of *DUF24 kinase* and *α-glucosidase* transcripts (**Fig. 10C**).

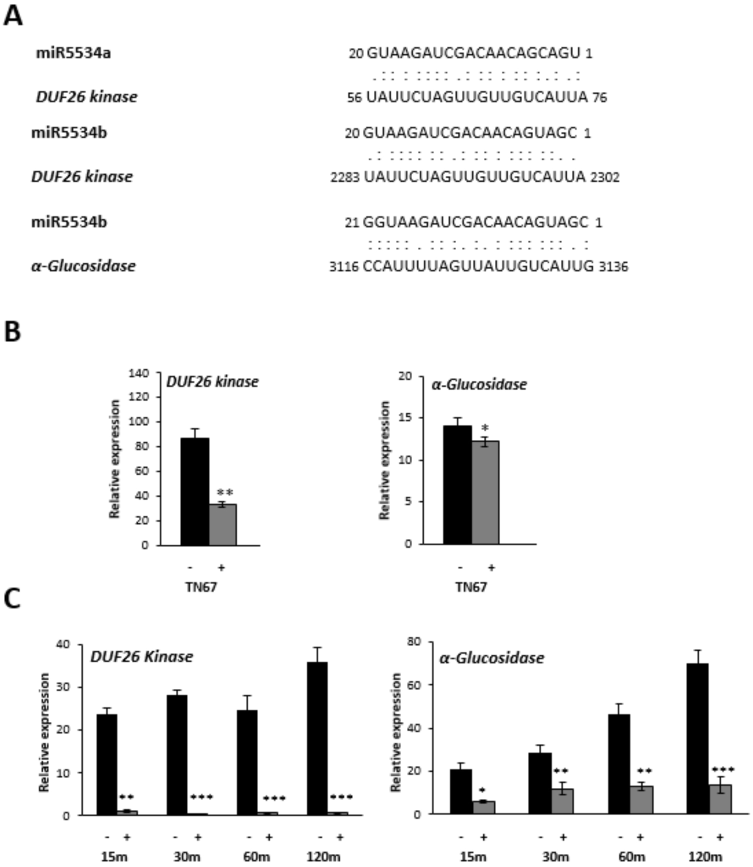


Figure 10. Transcript accumulation of target genes predicted for miR5534a-5534b, the *DUF26 kinase* and *α-glucosidase* genes, in wild-type rice plants. A. Alignement of miR5534a and miR5534b with their predicted target genes **B.** Expression was examined by RT-PCR in control (black bars) and *M. oryzae*-infected (72 hpi; grey bars) wild-type (TN67) plants. **C**. Expression in plants that have been treated, or not, with *M. oryzae* elicitors. Results from three biological replicates and at least 24 plants per condition are shown. Histograms show the mean ± SD (***, p-value ≤ 0.001 **, p-value ≤ 0.01; *, p-value ≤ 0.05, ANOVA test).

As summary, we have demonstrated that the rice polycistronic miR1861b-1861c and miR5534a-miR5534b respond to *M. oryzae* infection and elicitor treatment, supporting that they mediate PTI responses. T-DNA activation tagging of each one polycistronic miRNA confers resistance to *M. oryzae* infection supporting that miR1861b-1861c and miR5534a-5534b are positive regulators of rice immunity against the blast fungus.

References

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

Rayapuram, C., Jensen, M. K., Maiser, F., Shanir, J. V., Hornshøj, H., Rung, J. H., Gregersen, P. L., Schweizer, P., Collinge, D. B., and Lyngkjær, M. F. 2012. Regulation of basal resistance by a powdery mildew-induced cysteine-rich receptor-like protein kinase in barley. Mol. Plant Pathol. 13:135–147

Wrzaczek, M., Brosché, M., Salojärvi, J., Kangasjärvi, S., Idänheimo, N., Mersmann, S., Robatzek, S., Karpiński, S., Karpińska, B., and Kangasjärvi, J. 2010. Transcriptional regulation of the CRK/DUF26 group of receptor-like protein kinases by ozone and plant hormones in Arabidopsis. BMC Plant Biol. 10:95

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