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Role of microRNAs in Plant innate immunity

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PhD Thesis

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Dissertation presented by Patricia Baldrich for the degree of Doctor of Biology and Plant Biotechnology by Universitat Autònoma de Barcelona. This work was performed in Center for research in Agricultural Genomics.

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"Science is a way of life. Science is a perspective. Science is the process that takes us from confusion to understanding in a manner that's precise, predictive and reliable - a transformation, for those lucky enough to experience it, that is empowering and emotional."

Brian Greene

Aknowledments

Esta es obviamente la parte más divertida de la tesis, es la parte que discretamente todos leen en diagonal, buscando sus nombres. También es, como si dijéramos, la parte de los cotilleos de la tesis. Con esto no le estoy quitando importancia, evidentemente, también es la parte que se escribe de corazón, la parte en la que se dan las gracias a todos esas personas que de una manera u otra son parte indispensable de la tesis.

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General introduction

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General introduction

Small RNAs in plants

Small RNAs (sRNAs) are short non-coding RNAs that guide gene silencing in most eukaryotes (Baulcombe 2004; Vaucheret 2006). Plants have two main classes of sRNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs), which are distinguished by their mode of biogenesis and mechanisms of action. Plant miRNAs are transcribed from *MIR* genes by the RNA polymerase II enzyme as long single-stranded RNA precursors with unique stem-loop structures, the primary-miRNAs (or pri-miRNAs). The pri-miRNAs are then processed in two steps by RNAse III DICER-LIKE proteins (DCL) to give rise to double stranded miRNA duplexes (miRNA/miRNA*, also named miRNA-5p/miRNA-3p duplexes), containing a 2-nt overhang at the 3´-end (Kurihara and Watanabe 2004). Other RNA-binding proteins that are necessary for plant miRNA biogenesis are the HYPONASTIC LEAVES 1 (HYL1), SERRATE (SE), NEGATIVE ON TATALESS 2 (NOT2), and DAWDLE (DDL) proteins (Kurihara et al. 2006; Lobbes et al. 2006; Yang et al. 2006; Dong et al. 2008; Machida and Yuan 2013; Wang et al. 2013). The miRNA processing proteins concentrate in discrete nuclear bodies, the so-called dicing bodies (D-bodies) (Fang and Spector 2007; Liu et al. 2012). Both strands of the miRNA/miRNA* duplex are methylated at their 3' end by HUA ENHANCER 1 (HEN1) and transported from the nucleus into the cytoplasm by HASTY (HST), and ortholog of mammalian Exportin 5 (Park et al. 2005). Once in the cytoplasm, mature miRNAs are loaded into the RNA-induced silencing complex (RISC) where ARGONAUTE1 is the core component. MiRNAs guide post-transcriptional gene silencing through sequence-specific cleavage or translational repression of target mRNAs.

In addition to miRNAs, plants also produce different classes of endogenous siRNAs, including trans-acting siRNAs (tasiRNAs), phased secondary siRNAs (phasiRNAs), natural antisense siRNAs (natsiRNAs) and the most abundant one, heterochromatic siRNAs (hcsiRNAs). They differ from miRNAs in that they arise from double-stranded RNAs that originate through the action of RNA-dependent RNA polymerases (RDRs) (Chapman and Carrington 2007; Zhang et al. 2012). These

dsRNAs are then processed by DCL proteins (i.e. DCL2, DCL3 and DCL4) giving rise to distinct types of siRNAs that are also loaded into AGO-containing RISCs.

Production of each class of small RNAs has its own requirements for RDR and DCL proteins (Arikit et al. 2013; Axtell 2013).Thus, DCL1 is mainly involved in the generation of miRNAs, although alternative pathways for miRNA biogenesis involving DCL3 or DCL4 have also been described (Kurihara and Watanabe 2004; Rajagopalan et al. 2006; Vazquez et al. 2008; Arikit et al. 2013). RDR2 and DCL3 are preferentially used in the biogenesis of hcsiRNA whereas DCL4 acts mainly in the biogenesis of tasiRNAs in an RDR6-dependent manner (Xie et al. 2003; Peragine et al. 2004; Xie et al. 2005; Rajagopalan et al. 2006; Kasschau et al. 2007; Vazquez et al. 2008). A small subset of miRNAs guides the cleavage of TAS transcripts to produce ta-siRNAs. For instance, in Arabidopsis, miR173 targets TAS1, TAS2 (Yoshikawa et al. 2005), miR828 targets TAS4 (Rajagopalan et al. 2006), and miR390 targets TAS3 (Allen et al. 2005). As for AGO proteins, the core components of the RISC complex, AGO1 primarily binds miRNAs and AGO4 binds hc-siRNAs (Zilberman et al. 2003; Chan et al. 2004; Baumberger and Baulcombe 2005; Qi et al. 2006). Based on their nucleotide sequence, miRNAs are grouped into distinct families, each family comprising one or more members.

Numerous studies have demonstrated the crucial role of plant miRNAs in a wide range of developmental processes, including organ polarity and morphogenesis, flowering, shoot and root development, and hormone signaling, among others (Palatnik et al. 2003; Mallory et al. 2004; Mallory and Vaucheret 2006; Chen 2009; Wu et al. 2009a; Rubio-Somoza and Weigel 2011). In rice, the activity of certain miRNAs significantly controls traits of agronomic importance, such as tiller growth, early flowering, panicle and grain production (Miura et al. 2010; Wang et al. 2012; Zhang et al. 2013). There are also reports indicating that miRNAs are involved in the plant response to abiotic stress, including drought, salinity, cold, heat, oxidative stress and nutrient deficiency (Chiou et al. 2006; Pant et al. 2008; Liang and Yu 2010; Hackenberg et al. 2013). New insights into miRNA function came with the discovery that miR393 is involved in disease resistance in Arabidopsis plants (Navarro et al. 2006; Staiger et al. 2013; Yang and Huang 2014).

Evidence also exists on cell-to-cell and long distance movement of certain miRNAs (Marín-González and Suárez-López 2012). Cell-to-cell movement appears to occur via plasmodesmata, whereas long distance movement occurs via the vascular

system, in particular the phloem. Cell-to-cell movement of miRNAs was first demonstrated for miR156/166 in maize and Arabidopsis (Juarez et al. 2004; Kidner and Martienssen 2004). MiR399 represents another example of miRNA movement in plants, this particular miRNA being involved in phosphate homeostasis (Fujii et al. 2005; Hackenberg et al. 2013). Thus, reciprocal grafting experiments between wild-type and miR399-overexpressing Arabidopsis plants demonstrated the movement of miR399 from shoots to roots (Lin and Chiou 2008). In this way, miR399 acts as a long-distance signal that suppresses target gene expression in distal organs under Pi deficiency. Not only miR399, but also other miRNAs have been found in phloem sap of diverse plant species (Pant et al. 2008; Marín-González and Suárez-López 2012). In other studies, it was demonstrated that siRNAs are also mobile molecules in Arabidopsis (Chitwood et al. 2009). Here, the authors described the intercellular movement of tasiR-ARFs, particularly tasiR-ARFs, from their defined source of biogenesis on the upper (adaxial) side of leaves to the lower (abaxial) side (Chitwood et al. 2009). This creates a gradient on small RNAs that contributes to specification of the adaxial/abaxial polarity in leaves. TasiR-ARFs. Production of tasi-ARFs requires the specific action of miR390 for the initial cleavage of the non-coding TAS3 transcripts which then triggers the production of tasiRNAs targeting members of the AUXIN RESPONSE FACTOR (ARF) transcription factor family. At present, however, the molecular mechanisms that determine miRNA and siRNA movement potentially serving as mobile signals in plants remains elusive. Similarly, the extent to which that movement might have a role in plant immunity is unknown.

Here, we discuss recent insights into miRNAs and other small RNAs involved in the plant immunity, with a particular focus on rice. Compared to what is known in Arabidopsis, less information is available about the relevance of small RNA-based regulation of gene expression in the rice response to pathogen infection. A brief presentation of the involvement of components of the small RNA biogenesis and functioning on disease resistance is also included.

Plant pathogens

Rice and rice pathogens

Rice (*Oryza sativa*) is the most widely consumed staple food for a large part of the world's human population, providing a major portion of calories in human diet. One of the major factors limiting rice production is the occurrence of diseases caused by various fungal, bacterial and viral pathogens. Considering the annual average population growth rate, rice production will need to at least double by the year 2050. A practical means of achieving greater yields is to develop strategies to minimize losses due to diseases. This goal can be met only by integrating conventional breeding programs and marker-assisted selection (MAS) with modern biotechnological approaches (e.g. genetic engineering).

Blast caused by the ascomycete fungus *Magnaporthe oryzae* is the most devastating disease of cultivated rice worldwide (Skamnioti and Gurr 2009; Wilson and Talbot 2009). Based on its scientific and economic relevance, rice blast was considered the most important disease caused by fungi in plants (Dean et al. 2012). Sheath blight, caused by the fungus *Rhizoctonia solani*, is the second most devastating fungal disease of rice (Banniza and Holderness 2001). Bakanae ("foolish seedling" in Japanese) is also a common disease in rice which is caused by the fungus *Fusarium* spp. *(Gibberella fujikuroi* species complex) (Wulff et al. 2010).

Concerning bacterial diseases, bacterial blight and bacterial leaf streak (caused by *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv *oryzicola*, respectively) are the major bacterial diseases in rice (Niño-Liu et al. 2006). Other bacterial pathogens of rice are *Dickeya zeae* (previously known as *Erwinia chrysanthemi* pv. *zeae*), the causal agent of bacterial foot rot (Pu et al. 2012), and *Burkholderia glumae* which causes bacterial panicle blight of rice which is becoming an important disease problem in global rice production (Ham et al. 2011).

Diseases caused by viruses have become increasingly important in many rice-growing areas. The distribution of each virus is generally restricted to one of the continents in which rice is grown, *e.g.*, Rice hojablanca virus (RHBH) in South-America, and Rice Yellow Mottle Virus (RYMV) in Africa. Rice stripe virus (RSV), Rice tungro viruses and Rice dwarf virus (RTVB, RTSV and RDV) are found in Asia.

Arabidopsis and Arabidopsis pathogens

Arabidopsis has around 750 accessions collected from all around the world which are available in the two major seed stock centers. It has a small genome (125Mb) distributed in five different chromosomes. *Arabidopsis thaliana* was the first plant species with a complete genome sequenced (reported in 2000, Arabidopsis et al. 2000). It also has a short life cycle, efficient transformation protocols and a large collection of mutants and ESTs are already available in different public resources. All this characteristics make Arabidopsis the main model plant for molecular biology and genetics.

Different pathosystems have been described in *Arabidopsis thaliana*. Furthermore, pathogens from closely related species or distant plants have been assayed in Arabidopsis to investigate plant defense responses to pathogen infection. Concerning fungal pathogens, a large number of them have been reported to infect Arabidopsis, including obligate biotrophs (*Puccinia sp.*), hemibiotrphs (*Phytophtora sp., Colletotrichum sp.*) and necrotrophs (*Botrytis sp., Fusarioum sp., Plectosphaerella sp.*). (Dean et al. 2012)

For plant-bacterial interactions, perhaps the best characterized one is that with *Pseudomonas syringae*. The second most important plant bacteria pathogen is *Ralstonia solanacearum*, a high destructive bacterial pathogen for several plant species (Mansfield et al. 2012)

Plant innate immunity

Plants have evolved a multilayered innate immune system to defend themselves against pathogens. The first line of defense occurs through recognition of conserved Pathogen Associated Molecular Patterns (PAMPs) by host Pattern-Recognition Receptors (PRR). Sensing PAMPs triggers a general defense response referred to as PAMP-triggered immunity (PTI), which operates against most pathogens. PTI components include deposition of callose, production of reactive oxygen species (ROS), activation of protein phosphorylation/dephosphorylation processes and accumulation of Pathogenesis-related proteins (PRs), among others (Jones and Dangl 2006; Boller and He 2009). To counteract this innate defense, pathogens produce effectors that suppress PTI.

In turn, many plants have evolved another layer of immunity triggered by Resistance (R) proteins that responds to pathogen effector proteins. This type of immunity is called Effector-Trigered Immunity (ETI) and relies on the specific recognition of microbial effectors (or host proteins modified by effectors) by proteins encoded by *R* genes. Thus, this recognition triggers a rapid and effective host defense response. Based on the molecular structure of the encoded proteins R genes can be grouped into several classes. The most numerous *R*-gene class is represented by the members of the gene family that code for proteins containing a nucleotide-binding site (NBS) and leucine-reach repeats (LRRs). The presence of different domains at the *N*-terminal portion of the NBS-LRR proteins classifies these NBS-LRR gene products into two subgroups: the TIR-NBS-LRR proteins that contain the Toll/interleukin-1/receptor (TIR) domain, and the CC-NBS-LRR proteins that have a coiled-coil (CC) domain. Furthermore, the essential role of the phytohormones salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) in resistance to pathogens is also well established in plants (Pieterse et al. 2012; Denancé et al. 2013).

In rice, resistance to bacterial and fungal pathogens is conferred by both race-specific resistance genes (ETI) and basal resistance (PTI) (Liu et al. 2014). Concerning blast disease resistance, a broad array of blast resistance (*R*) genes has been described, and some of them have also been cloned and molecularly characterized. Some examples are the *Pib, Pi9, Pita, Pi2/Pizt, Pid2, Pi36, Pi37, Pik-m, Pi5, Pit, Pid3, Pi21* genes. 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, rice improvement for durable resistance to blast based on *R* genes is difficult as most of the resistance genes break down in a few years because of the race specificity and the rapid change in pathogenicity of the blast fungus.

RNA silencing plays a critical role in plant resistance against viruses, with multiple silencing factors participating in antiviral defense. The RNA silencing-based antiviral defense involves the production of virus-derived small interfering RNAs and their association to effector proteins, which together drive degradation of viral RNA in a sequence-specific manner. Antiviral silencing starts with the DCL-dependent production of viral siRNAs (vsiRNAs) by DCL 4 and DCL2 activities (Hamilton and Baulcombe 1999; Parent et al. 2015). Although both RNA and DNA viruses are targeted by the RNA silencing degradation pathway, DNA viruses can also be targeted

by RNA-directed viral DNA methylation through the activity of DCL3 (Raja et al. 2014). AGO proteins are essential in these processes, AGO1 and AGO2 acting in a synergic manner in viral RNA degradation and AGO4 in viral DNA methylation (Mallory and Vaucheret 2010; Wang et al. 2011). In addition to this, cleaved viral transcripts are recognized by a host RDR and secondary vsiRNAs are produced. This amplification mechanism can boost vsiRNA production, and systemically protect the plant against the same virus at distant sites.

In order to circumvent antiviral RNA silencing, plant viruses activate their counter-defense mechanisms by expressing RNA-silencing suppressors or adopting silencing-resistant RNA structures. Since the first identified viral RNA silencing suppressor (VRS) (Ding et al. 1996), several VRS have been characterized and are now considered to be essential for viral life cycle. Some VRS acting at dsRNA or DCL levels, such as the *Rice stripe virus* NS3 suppressor that binds small RNAs (Xiong et al. 2009) or the *Rice Yellow Mottle Virus* P1 suppressor that inhibits DCL4 dependent pathways. Other VRS can modulate AGO levels, for example by promoting miR168 transcription that results in a down regulation of AGO1 (Várallyay et al. 2010). There are also VRS acting at the RDR6 level (i.e. the *Rice Dwarf Virus* PNS10 suppressor) or the SGS3 level (i.e. the *Rice Stripe Virus* P2 suppressor) (Ren et al. 2010; Du et al. 2011b).

Endogenous Small RNAs in plant immunity

Traditionally, the regulation of plant immune responses has been studied at the transcriptional level. However, several studies support that plants also use post-transcriptional regulation of immune responses triggered by pathogen infection and that small RNA-based regulatory pathways are essential in reprogramming gene expression in plant disease resistance (Navarro et al. 2006; Ruiz-Ferrer and Voinnet 2009; Katiyar-Agarwal and Jin 2010; Shivaprasad et al. 2012; Staiger et al. 2013).

Evidence for miRNAs controlling pathogen resistance came first in Arabidopsis plants where perception of flg22, a peptide derived from the general elicitor flagellin, causes an increase in miR393 accumulation which in turn negatively regulates transcripts for F-box auxin receptors. The miR393-mediated expression of auxin signalling results in bacterial resistance (Navarro et al. 2006). This study clearly

established a link between miRNA functioning, hormone signalling and immunity in plants. Since then, the contribution of small RNAs has been documented in different pathosystems (Padmanabhan et al. 2009; Ruiz-Ferrer and Voinnet 2009; Ding 2010; Jang-Kyun Seo et al. 2013; Weiberg et al. 2014; Lee and Yeom 2015). Some of the pathogen-associated miRNAs might be specifically required during ETI responses, whereas others might function in PTI.

Concerning ETI, distinct miRNAs that guide cleavage of *R* genes have been described in different plant species over the past few years. For instance, a miRNA superfamily comprising miR482 and miR2118 that target NBS-LRR genes was described in tomato (Shivaprasad et al. 2012). In other studies, three abundant miRNA families, miR2109, miR472 and miR1510, were identified in the legume *Medicago truncatula.* (Zhai et al. 2011). These miRNAs target highly conserved, protein-coding motifs in diverse members of the NB-LRR class of R genes, and also trigger the production of phased, secondary siRNAs (phasiRNAs). Li et al. (2012) identified two tobacco miRNAs (nta-miR6019 and nta-miR6020) that guide cleavage of transcripts of the TIR-NB-LRR immune receptor *N* that confers resistance to tobacco mosaic virus (TMV). Co-expression of *N* with nta-miR6019 and nta-miR6020 resulted in attenuation of *N*-mediated resistance to TMV, further supporting that these miRNAs have functional roles in *N* gene regulation. In this study, the authors demonstrated that cleavage of *N* transcripts by nta-miR6019 also triggers the production of secondary siRNAs in phase with the miR6019 cleavage site. Taken together, these findings suggest the existence of a miRNA-mediated regulation of disease resistance genes, and that some of these regulatory miRNAs trigger the production of secondary siRNAs from their *R* gene targets.

During the last years, the adoption of deep sequencing technologies has significantly contributed to uncover multiple miRNAs, as well as other endogenous sRNAs, in plants. The miRBase registry (http://www.mirbase.org) represents the central online repository for microRNA (miRNA) nomenclature, sequence data, annotation and target prediction. However, the discovery of new miRNAs in rice might have reached a plateau (**Figure 1**).

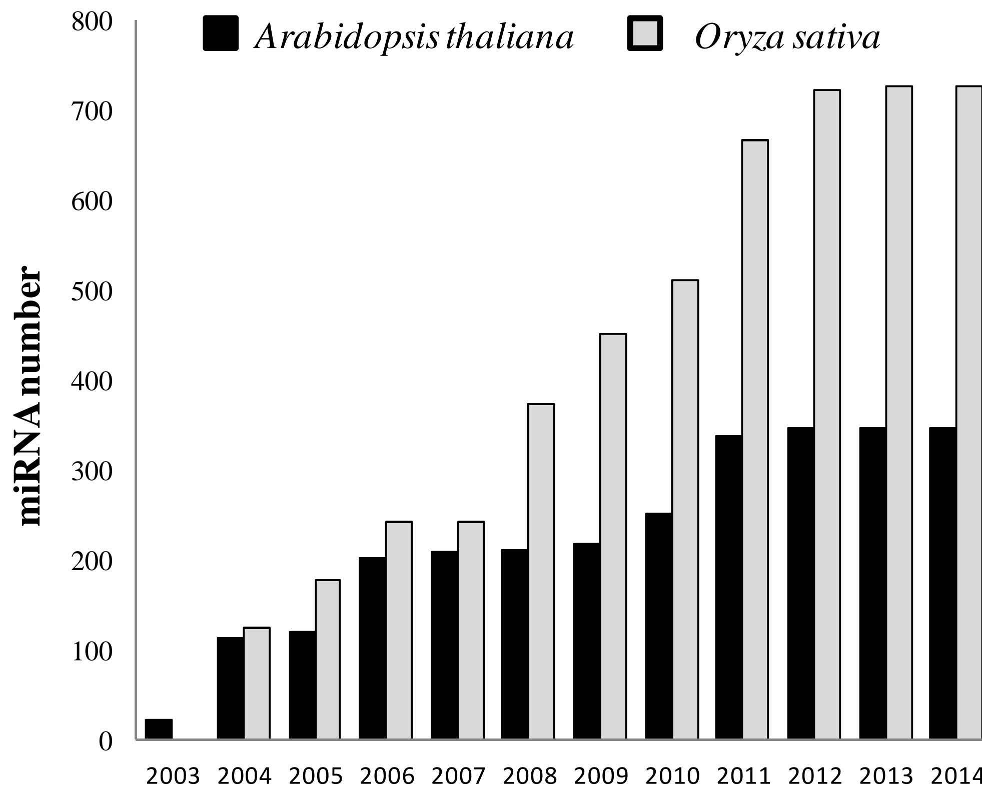


Figure 1. Evolution of miRNA discovery. Known miRNAs in *A. thaliana* and *O. sativa* were taken from miRBase (http: http://www.mirbase.org; (Kozomara and Griffiths-Jones 2014)) up to release 21 (2014).

In the literature, there are many examples that describe massive and dynamic alterations in miRNA populations during pathogen infection in plant species, including rice (Du et al. 2011a; Campo et al. 2013; Li et al. 2013; Sun et al. 2014; Baldrich et al. 2015). For information on pathogen-regulated miRNAs in different plant species we refer to recent reviews by Gupta et al. (2014), Balmer and Mauch-Mani (2013). However, although a substantial fraction of the miRNA transcriptome of a plant has been found to be pathogen-responsive, the functional characterization of most of these miRNAs in disease resistance remains unknown.

An important number of pathogen-regulated miRNAs are known to target genes that are important regulators in developmental processes and hormone signaling which might also have strong effects on disease resistance. Our understanding of the functional roles of miRNAs in plant disease resistance is, however, far less than that in plant development. Presumably, the ability of a plant to adjust developmental programs during infection conditions might enhance the plant's ability to escape from, resist or compensate for disease. Furthermore, because miRNAs provide the quantitative

regulation of target gene expression, rather than on-off regulations, the observed dynamic responses on miRNA accumulation would provide the fine-tuning of gene expression in different physiological processes contributing to disease resistance. A priori, pathogen-induced miRNAs might target negative regulators of the plant response to pathogen infection whereas pathogen-repressed miRNAs might target positive regulators. Then, a pathogen-regulated accumulation of miRNAs has the potential to alter the outcome of the targeted pathway which, in turn, would have an effect in pathogen resistance. As an example, miR160a overexpression enhances callose deposition in bacterial resistance in Arabidopsis, this particular miRNA acting as a positive regulator of PAMP-induced responses. In contrast, miR398 and miR773 negatively regulate callose deposition and their overexpression promotes bacterial growth (Li et al. 2010a).

On the other hand, PTI and ETI have been historically considered as protein-based defense mechanisms acting in antibacterial and antifungal resistance, largely independent from antiviral RNA silencing (RNA-based defense mechanisms). However, a recent body of evidence indicates that this view requires considerable amendment, because endogenous sRNAs are now emerging as key regulators of PTI and ETI signaling. MiRNAs for which functional evidence exists for their involvement in disease resistance in different pathosystems are presented in **Figure 2**.

As was originally discovered in plant-virus interactions, bacteria and oomycetes are also able to produce suppressors of RNA silencing as effector proteins (Navarro et al. 2008; Qiao et al. 2013). Under this scenario, mechanisms of RNA silencing suppression by plant pathogens can be considered as part of the molecular arms race between plant pathogens and their hosts. Recently reviews Pumplin and Voinnet 2013 and Weiberg et al. 2014 illustrates how certain pathogens are able to manipulate host silencing pathways to counteract plant defense responses.

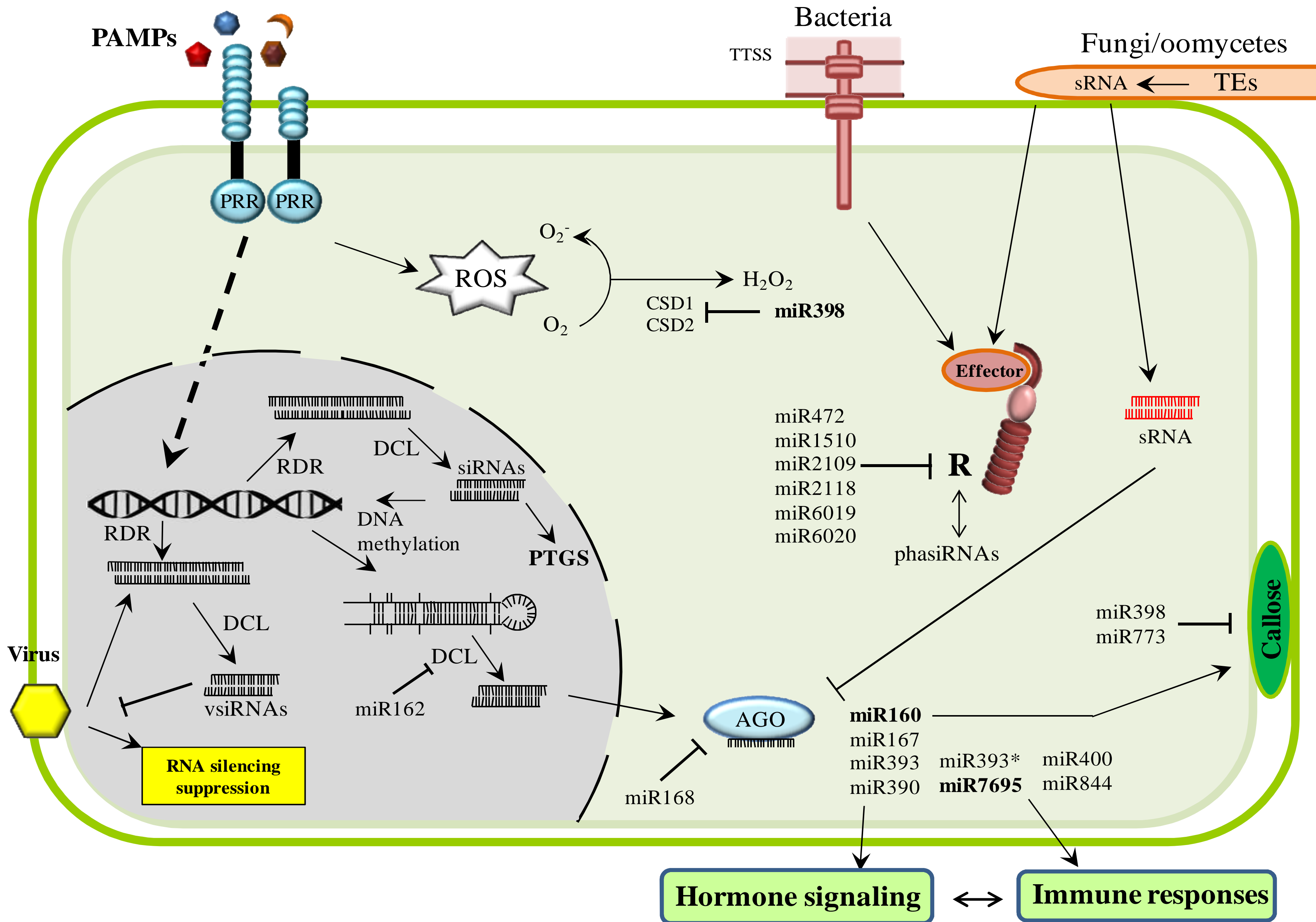
During recent years, great efforts have been made for the characterization of miRNAs in rice. Most of these studies focused on the characterization of miRNAs accumulating in different tissues and/or at developmental stages or in response to abiotic stresses, but miRNAs related to pathogen infection are less characterized. The small RNA population from seedlings, shoot, root grown under normal conditions, as well as in plants subjected to abiotic stresses (e.g. drought, salt, temperature and nutrient

stress) is documented (Jeong et al. 2011). This study, not only provided a comprehensive catalogue of miRNAs, but also revealed a differential regulation in the expression of members of the same miRNA family in different organs.

Rice is actually the second plant species in number of annotated miRNAs in miRBase, only after *Medicago truncatula*. This reflects the important gap that occurs between identified miRNAs and their functions in rice, as the biological function for most of the annotated rice miRNAs remains unknown.

On the other hand, the first plant microRNA annotated in the miRBase was Arabidopsis, 33 mature microRNAs, distributed in 14 families, were annotated (Reinhart et al. 2002).

Figure 2. Functionally characterized miRNAs in relation to disease resistance. Most of them have been characterized in Arabidopsis plants infected with *Pseudomonas syringae*. In rice, the involvement of miR160, miR398 and miR7695 is documented (Campo et al. 2013; Li et al. 2013). In the case of miR393, the two sRNAs of the duplex are functional during pathogen infection (miR393 targets *TIR1*, whereas miR393* regulates MEM12, a Golgi-localised SNARE protein that modulates exocytosis of antimicrobial PR1 proteins in Arabidopsis (Zhang et al. 2011)). PAMPs, Pathogen-associated molecular pattern; PRR, Pattern recognition receptor; TTSS, Type three secretion system; RDR, RNA-dependent RNA polymerase; DCL, Dicer-like protein; AGO, Argonaute; sRNA, small RNA; vsiRNA, virus-derived small interfering RNA; TEs, Transposons; ROS, Reactive oxygen species; CDS, Copper/Zinc superoxide dismutase; PTGS, Post-transcriptional gene silencing.


PAMPs
PRR
PRR
Bacteria
TTSS
Fungi/oomycetes
sRNA
TEs
ROS
O2-
O2
H2O2
CSD1
CSD2
miR398
Effector
miR472
miR1510
miR2109
miR2118
miR6019
miR6020
R
phasiRNAs
sRNA
RDR
DCL
siRNAs
DNA methylation
PTGS
RDR
DCL
vsiRNAs
DCL
miR162
Virus
RNA silencing suppression
AGO
miR168
miR160
miR167
miR393
miR390
miR393*
miR7695
miR400
miR844
miR398
miR773
Callose
Hormone signaling
Immune responses

Role of miRNAs in Arabidopsis defense responses against pathogens.

Evidence exists for the involvement of several miRNAs in the defense response of Arabidopsis plants to pathogen infection. Indeed, the Arabidopsis miR393 was the first miRNA for which a role in disease resistance was demonstrated (Navarro et al. 2006). The accumulation of diverse miRNAs have been shown to be regulated by infection in Arabidopsis (for a review see Ruiz-Ferrer and Voinnet 2009). In the last year, two different miRNAs were associated to pathogen attack in Arabidopsis, miR400 and miR844. The miR400 and miR844 target the different Pentatricopeptide Repeat Proteins, PPR1 and PPR2 (Park et al. 2014) and cytidinephosphate diacylglycerol synthase 3 (CDS3) (Lee et al. 2015). PPR proteins play a role in posttranscriptional regulation of gene expression by controlling RNA metabolism (Ichinose et al. 2013). As for CDS3 proteins, they catalyze the formation of cytidinediphosphate diacylglycerol (CDP-DAG), an essential component for cellular functions. Overexpression of miR400 and miR844 in Arabidopsis causes an increased susceptibility to the bacterial pathogen *Pseudomonas syringae pv. tomato* DC3000, as well as to the fungal pathogen *Botrytis cinerea*. However, the mechanisms by which miR400 and miR844, and their target genes, regulate defense responses in Arabidopsis still needs to be elucidated.

Role of miRNAs in rice defense responses against fungal pathogens.

In the literature, several reports are found that describe transcriptome alterations in rice plants infected by the rice blast fungus, during both compatible and incompatible interactions (Vergne et al. 2007; Bagnaresi et al. 2012; Campo et al. 2013; Baldrich et al. 2015). Also, the mixed transcriptome of rice and blast fungus was obtained from infected rice leaf blades using RNA-Seq which allowed both rice and *in planta* fungal gene expression. These studies revealed that a plethora of miRNAs are regulated during infection of rice plants with the fungal pathogen *M. Oryzae* (Kawahara et al. 2012). Very recently, deep sequencing of small RNA libraries in combination with degradome analyses revealed regulatory networks enriched in rice leaves that have been treated with elicitors obtained from the rice blast fungus (Baldrich et al. 2015). Thus, an important number of miRNA/target gene pairs involved in small RNA pathways, including miRNA, heterochromatic and trans-acting siRNA pathways, and hormone signaling were identified in elicitor-treated rice leaves.

Despite the notable number of miRNAs that are regulated during *M. oryzae* infection, the exact role of these pathogen-responsive miRNAs in rice immunity remains elusive. Only recently, a functional role of distinct rice miRNAs in resistance to the blast fungus *M. oryzae* has been demonstrated. They are miR160a, miR398b and miR7695 (Campo et al. 2013; Li et al. 2013).
In plants, miR160 and miR398 are conserved, and have conserved target genes. MiR160 targets Auxin Response Factors (ARFs) involved in auxin signaling in plants. Auxins have a crucial role not only in development (Teale et al. 2006), but also in the control of plant immune responses (Spoel and Dong 2008; Yang et al. 2013b). As previously mentioned, the Arabidopsis miR393 contributes to antibacterial resistance by repressing auxin signaling (Navarro et al. 2006). Also in Arabidopsis, repression of the auxin response pathway increased susceptibility to necrotrophic fungi (Llorente et al 2008 Mol Plant 1, 496-). In rice, auxin homeostasis regulates the expression of rice defense genes and resistance to the blast fungus (Domingo et al. 2009). As for miR398, this miRNA targets two Cu/Zn superoxide dismutase genes (CSD1 and CSD2) and a copper chaperone for superoxide dismutase (CCS1) (Wu et al. 2009b; Zhou et al. 2010; Li et al. 2010b). Cu/Zn CSDs are a group of metalloenzymes which act as scavengers of reactive oxygen species (ROS), thus, protecting plants against oxidative stress (Beyer et al. 1991). To note, transgenic rice plants overexpressing either miR160a or miR398b displayed enhanced resistance to *M. oryzae* (Li et al. 2013). The expression of defense-related rice genes (i.e. PR1 and PR10) was found to be up-regulated in the miR160a and miR398b overexpression plants.

MiR7695 is a rice-specific miRNA that negatively regulates the accumulation of an alternatively spliced transcript of the *OsNramp* (Natural resistance-associated macrophage protein 6) gene. Most importantly, overexpression of miR7695 confers resistance to rice blast fungus (Campo et al. 2013). Concerning the biochemical function of the miR7695 target gene, NRAMP proteins are known to be involved in the transport of divalent metals and maintenance of metal homeostasis in a wide range of organisms, including plants (Cellier et al. 1996). The finding that miR7695 positively regulate immunity against the rice blast fungus raises interesting questions concerning the role of *nramp* genes in disease resistance. In Arabidopsis, NRAMP3 and, to a lesser extent, NRAMP4 participate in iron mobilization and appear to be involved in resistance against the bacterial pathogen *Erwinia chrysanthemi* (Lanquar et al. 2005;

Segond et al. 2009). At present, however, the biological significance of a miR7695-mediated regulation of *OsNramp6* in the context of rice immunity remains unknown. Additionally, miR7695 represents a recently evolved miRNA that experienced natural and domestication events during rice evolution (Campo et al. 2013). Collectively, these findings illustrate the potential of miRNAs as transgenes for disease resistance in rice.

Small RNAs in rice/virus interactions.

Several studies have demonstrated that plant miRNAs respond to virus infection, and that these virus-responsive miRNAs are associated with viral symptoms or virus pathogenicity (Bazzini et al. 2007; Xiao et al. 2014). High-throughput sequencing has been also used to monitor alterations in the expression of small RNAs, including miRNAs, in virally-infected rice plants (Du et al. 2011a; Guo et al. 2012; Sun et al. 2014). For instance, small RNA profiling of rice plants infected by two distinct viruses, Rice Dwarf virus (RDV) and rice stripe virus (RSV), demonstrated that infection by one or another virus had distinct impacts on the rice small RNA population (Du et al. 2011). Some conserved miRNAs respond to Rice Stripe Virus (RSV) but do not respond to Rice Dwarf Virus (RDV), and the other way around. The ten most abundant miRNAs accumulating in RSV-infected rice were miR168, 156, 396, 159, 535, 166, 172, 167, 528 and 444. In this study, the authors also described that RSV infection, but not DRV infection, enhanced the accumulation of some rice miRNA*s (but not their corresponding miRNAs) as well as the accumulation of phased siRNAs from particular miRNA precursors.

MiRNAs that are responsive to infection by RSV (Guo et al. 2012) or by the Rice black streaked dwarf virus (RBSDV) in rice plants have been described (Sun et al. 2014). In particular, analysis of RBSDV-infected rice plants revealed important alterations in the accumulation of 14 miRNAs in leaves and 16 miRNAs in roots. Although some miRNAs exhibited a similar response to RBSDV infection in roots and leaves, many miRNAs had different expression patterns depending on the tissue, leaves and roots (Sun et al. 2014). Studies in rice plants infected with Rice stripe virus allowed the identification of novel miRNAs in RSV-infected rice (Guo et al. 2012).

Role of small RNA pathway components in plant immunity

In addition to sRNAs themselves, several components of the sRNA pathway also play a role in disease resistance. For instance, the Arabidopsis dcl1-9 mutant (defective in miRNA biogenesis) as well as ago1-25 and ago1-27 mutants (defective in miRNA activity) are impaired in PTI and display enhanced susceptibility to bacterial infection whereas the Arabidopsis dcl1-7 mutant is more susceptible to the fungal pathogen *Botrytis cinerea* (Navarro et al. 2008). Also in Arabidopsis, the *rdr6* mutant is more susceptible to bacterial and fungal infection (Katiyar-Agarwal et al. 2006; Ellendorff et al. 2009). As previously mentioned, RDRs are responsible of generating dsRNAs which are subsequently processed into sRNAs by DCLs.

Less is known about the contribution of sRNA components in rice immunity. Only, *dcl2* is known to be up-regulated whereas *dcl3a* and *dcl3b* are down-regulated during RSV infection. However, whether *dcl2* and *dcl3* are involved in virus defense in rice still needs to be elucidated (Du et al. 2011b). Concerning RDRs, it was reported that silencing of rice *rdr6* enhances susceptibility to RSV infection (Jiang et al. 2012).

On the other hand, several studies have been conducted and reported in the literature on the involvement of AGO1 in plant antiviral defense (Takeda et al. 2008; Várallyay et al. 2010; Harvey et al. 2011; Scholthof et al. 2011; Wang et al. 2011). Similar to miRNAs, viral-derived siRNAs are incorporated into AGO1-containing RISC leading to inactivation of viral RNAs by cleavage. In turn, viruses may encode viral suppressors that impair AGO1 functionality (reviewed by Burgyan and Havelda, 2011). In plants, AGO1 homeostasis is in part coordinated by a feedback mechanism that entails miR168-guided cleavage of AGO1 and posttranscriptional stabilization of miR168 by AGO1 (Vaucheret et al. 2006). In other studies, it was demonstrated that the Arabidopsis AGO1 is required for *Verticillium* pathogenicity (Ellendorff et al. 2009). It is also demonstrated that AGO2 is involved in pathogen immunity by binding miR393* (Zhang et al. 2011). In rice, AGO18 was found to be induced upon infection by RSV and RDV, thus, sequestering miR168 and allowing the activation of AGO1 (Wu et al. 2015).

Pathogen-derived sRNAs in pathogenicity

The relevance of pathogen-derived sRNAs in plant immunity has been recognized only recently. As previously mentioned, certain pathogens produce effectors that suppress host plant immunity as part of their virulence strategy. Most effectors so far characterized are proteins. Interestingly, studies in Arabidopsis and tomato plants infected by the fungus *Botrytis cinerea* revealed that pathogen-derived small RNAs might also act as effectors to inhibit host immunity (Weiberg et al. 2013; Weiberg et al. 2014). *B. cinerea* is the causal agent of gray mold disease in numerous plant species. As part of the fungal virulence strategy, *Botrytis cinerea* transfer small RNAs into plant cells. These small RNAs are able to hijack the host RNA interference (RNAi) machinery by binding to AGO1 to selectively target transcripts of host defense genes, thus, suppressing host immunity (Weiberg et al. 2013). *B. cinerea* small RNA effectors are predominantly transcribed from transposable elements. The discovery of small RNAs acting as effector molecules that suppress host immunity demonstrates a naturally occurring cross-kingdom RNA interference mechanism acting during pathogenesis.

Oomycete plant pathogens, such as *Phytophthora sojae*, also use suppression of RNA silencing as a strategy to cause disease (Qiao et al. 2013). Here, effectors from the oomycete pathogen were found to suppress RNA silencing by inhibiting the biogenesis of sRNAs. Altogether, these findings are consistent with RNA silencing mechanisms having key roles in plant immunity.

Although prokaryotes do not possess DCL proteins, regulatory noncoding sRNAs have also emerged as important components in bacterial virulence which have structural features and mechanism of action completely different to those found in fungal and oomycete pathogens. Thus, bacterial noncoding sRNAs operate with RNA-binding protein complexes, as it is the case for the Clustered regulatory interspaced short palindromic repeat /CRISPR-associated (Cas) system (CRISPR-Cas) complex. CRISPR/Cas systems function in bacterial defense against invading foreign nucleic acids derived from bacteriophages or exogenous plasmids. CRISPR loci consists of contiguous, highly conserved repeat sequences and variable spacers (CRISPR RNAs, or crRNAs) that recognize their targets. After viral challenge, bacteria integrate new spacers derived from phage genomic sequences. Cas genes encode proteins that help to recognize and integrate foreign DNA into a new repeat-spacer unit in the CRISPR locus

and mediate target degradation. Thus, the CRISPR/Cas system represents a unique prokaryotic adaptive immune system, similar to eukaryotic RNA interference defense against genome invading DNA and RNA elements, such as viruses (Barrangou et al. 2007). However, although small RNA profiling studies and genome sequencing have identified potential sRNAs in phytopathogenic bacteria (i.e. *Xanthomonas oryzae* pv *oryzae* (Liang et al. 2011)), their function in pathogenesis in plants remain to be determined.

Applications of miRNA-based strategies for rice improvement

Nowadays, miRNA-based genetic modification technology has emerged as an attractive approach not only in functional genomics but also for crop improvement. The use of miRNA-based technology provides a smart solution for sequence-specific cleavage of any designated target transcript with a great potential for biotechnological approaches for targeted gene silencing. In line with this, several investigations have shown that distinct miRNAs can highly affect agricultural traits in rice (Miura et al. 2010; Wang et al. 2012; Zhang et al. 2013). For instance, miR397 overexpression was described to improve rice yield by increasing grain size and promoting panicle branching (Zhang et al. 2013), whereas rice plants overexpressing miR319 had wider leaf blades and enhanced cold tolerance (Yang et al. 2013a). For additional information on rice miRNAs that control agronomic traits such as yield, quality and stress tolerance, and their use in rice biotechnology, we refer to articles by Zhou and Luo (2013) and Zheng and Qu (2015). Furthermore, recent progress in the development of strategies for crop improvement based on sRNA-mediated gene regulation have been recently reviewed by Kamthan et al. (2015). In general, miRNAs can be considered a good resource for plant genetic engineering and crop improvement.

In addition to miRNAs themselves, there is also the possibility of manipulating miRNA target genes for plant trait modification. Altering the expression of a miRNA or its target gene might, however, lead to undesirable pleiotropic changes in plant morphology and/or development (i.e. multiple targets might exist for a particular miRNA). Clearly, a better knowledge of the mechanism governing miRNA function is required to avoid potential undesirable trade-off effects in transgenic plants with altered

expression of miRNAs. The growing understanding of miRNAs playing a role in the defense response of rice plants to pathogen infection will offer new possibilities in designing novel and efficient strategies for disease resistance. Given that miRNAs can act as regulators of gene expression in ETI and PTI responses, they represent a good resource for the development of disease resistant rice plants through genetic engineering.

However, social concerns raised by the use of genetic modified organisms (GMO) should be addressed and continuous scientific efforts are necessary for the development of methodologies for the safety assessment of transgenic rice. New technologies for precise, efficient gene targeting or genome editing such as the TALEN-based technique, have emerged as an alternative to transgenic methods. This technology makes possible introducing modifications in a plant genome, which are indistinguishable from those introduced by conventional mutagenesis (i.e. chemical or physical mutagenesis). To note, the TALEN (Transcription activator-like effector nuclease) technology arises from our knowledge on the infection process used by bacterial pathogens in plants. It exploits the activity of Transcription activator-like (TAL) effectors of *X. oryzae* that are delivered into the host cells via the bacterial type III secretion system acting as virulence factors during bacterial infection. The TALEN-based techniques have been successfully used to develop disease-resistant rice plants that do not contain foreign DNA by targeting the rice susceptibility gene *Os11N3* (Li et al. 2012b). More recently, a method based on the bacterial CRISPR/Cas9 system has been developed for genome editing and targeted mutagenesis in plants, including rice (Jiang et al. 2013; Miao et al. 2013; Zhang et al. 2014). Thus applications of research related with small non-coding RNAs have the potential to contribute to food safety by generating transgenic rice free of foreign DNA.

Artificial microRNAs (amiRNAs) also represents a miRNA-based strategy that exploit endogenous gene silencing mechanism to target any gene of interest while providing a highly specific approach for gene silencing in plants (Ossowski et al. 2008; Sablok et al. 2011). The amiRNAs can be created by exchanging the miRNA/miRNA* sequence within a miRNA precursor with a sequence designed to match the target gene. This technology has a great potential to be used for genetic functional studies as well as for genetic improvement in crops.

Another example that illustrates the usefulness of the miRNA-based technology for plant genetic engineering is the artificial target mimics system. Target mimicry is an

endogenous regulatory mechanism that plants use to negatively regulate the activity of specific miRNAs. MiRNA target mimicry was initially described in Arabidopsis by Franco-Zorrilla et al (2007). Here, an endogenous long non coding RNA (*IPS*, Induced by Phosphate Starvation 1) that binds to miR399 with a bulge between at the 10th-11th position of miR399 was identified (Franco-Zorrilla et al. 2007). Such pairing abolished the cleavage effect of miRNA on IPS1. In this way, IPS1 serves as a decoy for miR399 to interfere with the binding of miR399 to its target. Both endogenous decoy RNAs (miRNA target mimics) and engineered artificial target mimic RNAs can interfere with normal miRNA function in plants. The miRNA target mimicry has proven to be a useful tool to decipher the function of a miRNA of interest and can also be exploited to reduce the activity of miRNAs controlling traits of agronomic interest. In this respect, a transgenic approach was developed to improve panicle exertion of a rice cytoplasmic male sterile rice line by using a combination of artificial miRNA and artificial target mimic (Chen et al. 2013).

Finally, miRNAs have achieved considerable attention as potential biomarkers in plant stress responses (Bej and Basak 2014). However, before using miRNAs as genetic markers in breeding programs for disease resistance, a better knowledge of miRNA functioning and miRNA-regulated regulatory networks involved in rice defense responses to pathogen infection is needed. This information will be useful to define miRNAs as biomarkers for disease resistance traits and, hence, for improved crop productivity.

Future prospects

As plants are sessile in nature, they have developed different strategies to adapt and grow under rapidly changing environments. These strategies involve rapid and versatile reprogramming of gene expression in response to different types of stress, biotic and abiotic stress. Extensive data have shown that an important miRNA repertoire showed responsiveness to different types of stress. Whereas our knowledge about miRNA biogenesis and mechanisms governing miRNA/target gene interaction has greatly expanded in recent years, our understanding of miRNA-mediated processes underlying plant immunity is far from complete. Although the genome sequence of two subspecies of rice, *Oryza sativa* spp. *japonica* and *indica* ssp. are available (Goff et al.

2002; Yu et al. 2002; Kawahara et al. 2013), and miRNA profiling identified a large number of miRNAs in rice, the biological function for most of them remains unknown. Understanding miRNA-mediated regulation of gene expression in rice-pathogen interactions will have important implications in designing novel strategies for disease control which, in turn, might contribute to improve rice productivity. Transgenic expression of a miRNA (or their target), as well as the use of miRNA-based gene silencing approaches, will help in developing disease resistant rice plants. Taking into account that rice has been adopted as the model species in cereal genomics, efforts to identify rice miRNAs involved in disease resistance will benefit other cereal research programs.

Objectives

Objectives

Numerous studies focused on microRNA function on a wide range of developmental processes, hormone signaling or abiotic stress. Less information is, however, available on the role of small RNAs in the plant response to biotic stress. The main objective of this PhD thesis was to get new insights into small RNAs, particularly miRNAs, potentially contributing to plant innate immunity. The specific objectives were:

1. To identify Arabidopsis small RNAs, in particular miRNAs, whose expression is regulated by treatment with fungal elicitors. This study was carried out in Arabidopsis plants (Col 0) treated with elicitors obtained from the fungal pathogen *Fusarium oxysporum*. Results obtained in this part of the work are presented in Chapter I.

2. To identify rice miRNAs, and their corresponding target genes, regulated by treatment with fungal elicitors. This study was approached in the japonica rice cultivar *Nipponbare* using elictors obtained from the rice blast fungus *Magnaporthe oryzae*. As part of our study on rice miRNAs, it was also considered of interest to search for the presence of polycistronic miRNAs in the rice genome. Results obtained in these studies are presented in Chapters II and III.

Chapter I

Small RNA profiling reveals regulation of Arabidopsis miR168 and heterochromatic siRNA415 in response to fungal elicitors

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Abstract

Background: Small RNAs (smRNAs), including small interfering RNAs (siRNAs) and microRNAs (miRNAs), have emerged as important regulators of eukaryotic gene expression. In plants, miRNAs play critical roles in development, nutrient homeostasis and abiotic stress responses. Accumulating evidence also reveals that smRNAs are involved in plant immunity. Most studies on pathogen-regulated smRNAs have been conducted in Arabidopsis plants infected with the bacterial pathogen *Pseudomonas syringae*, or treated with the flagelin-derived elicitor peptide flg22 from *P. syringae*. This work investigates smRNAs that are regulated by elicitors from the fungus *Fusarium oxysporum* in Arabidopsis.

Results: Microarray analysis revealed alterations on the accumulation of a set of smRNAs in response to elicitor treatment, including miRNAs and small RNA sequences derived from massively parallel signature sequencing. Among the elicitor-regulated miRNAs was miR168 which regulates ARGONAUTE1, the core component of the RNA-induced silencing complex involved in miRNA functioning. Promoter analysis in transgenic Arabidopsis plants revealed transcriptional activation of *MIR168* by fungal elicitors. Furthermore, transgenic plants expressing a *GFP-miR168* sensor gene confirmed that the elicitor-induced miR168 is active. MiR823, targeting Chromomethylase3 (*CMT3*) involved in RNA-directed DNA methylation (RdDM) was also found to be regulated by fungal elicitors. In addition to known miRNAs, microarray analysis allowed the identification of an elicitor-inducible small RNA that was incorrectly annotated as a miRNA. Studies on Arabidopsis mutants impaired in small RNA biogenesis demonstrated that this smRNA, is a heterochromatic-siRNA (hc-siRNA) named as siRNA415. Hc-siRNAs are known to be involved in RNA-directed DNA methylation (RdDM). SiRNA415 is detected in several plant species.

Conclusion: Results here presented support a transcriptional regulatory mechanism underlying *MIR168* expression. This finding highlights the importance of miRNA functioning in adaptive processes of Arabidopsis plants to fungal infection. The results of this study also lay a foundation for the involvement of RdDM processes through the activity of siRNA415 and miR823 in mediating regulation of immune responses in Arabidopsis plants.

Introduction

The genomes of higher eukaryotes encode small RNAs (smRNAs) that direct transcriptional and post-transcriptional gene silencing (Baulcombe 2004; Jones-Rhoades et al. 2006; Vaucheret 2006). In plants, smRNAs can be categorized into two major classes including microRNAs (miRNAs) and small interfering RNAs (siRNAs) which are distinguished by their precursor molecules and different modes of biogenesis (Voinnet 2009; Axtell 2013; Rogers and Chen 2013).

MiRNAs are derived from primary miRNA (pri-miRNA) transcripts that form an imperfect fold-back structure (Xie et al. 2005a). The pri-miRNA is then processed in a two-step pathway by a Dicer-like (DCL) ribonuclease, typically DCL1, to produce miRNA/miRNA* duplexes which are methylated and exported to the cytoplasm (Xie et al. 2005a; Kurihara et al. 2006). Alternative pathways for miRNA biogenesis involving DCL3 or DCL4 have also been described (Rajagopalan et al. 2006; Vazquez et al. 2008). The miRNA is then selectively incorporated into the ARGONAUTE 1 (AGO1)-containing RNA-induced silencing complex (RISC) and thereby directs cleavage or translational inhibition of the target mRNA (Llave et al. 2002; Baumberger and Baulcombe 2005; Brodersen et al. 2008).

The second major class of smRNAs, includes siRNAs that are generated from long double-stranded RNAs (dsRNAs) resulting from the activity of RNA-dependent RNA polymerases (RDRs) that are sliced by DCL activities into siRNA duplexes (Xie et al. 2004; Axtell 2013). Thus, the most distinguishing feature of siRNA biogenesis is the requirement of RDR activity for generation of siRNA precursors, whereas miRNAs have single-stranded RNA precursors and do not require RDR activity for their biosynthesis. Plant siRNAs can be further categorized as heterochromatic siRNAs (hc-siRNAs; also referred to as repeat-associated siRNAs or ra-siRNAs), secondary siRNAs (including trans-acting siRNAs or ta-siRNAs), and natural antisense transcript-derived siRNAs (nat-siRNAs) (Axtell 2013). Production and function of each class of siRNAs has very consistent requirements for specific members of the DCL, RDR and AGO gene families. Whereas DCL2 is mainly involved in the generation of nat-siRNAs (Chan et al. 2004; Borsani et al. 2005), DCL3 is responsible for the processing of RDR2-generated dsRNA and gives rise to 24-nt hc-siRNAs (Xie et al. 2004; Kasschau et al.

2007). DCL4 acts mainly in the biogenesis of tasi-RNAs in an RDR6-dependent manner (Xie et al. 2005b; Rajagopalan et al. 2006). As for AGO proteins, the core components of the RISC complex, AGO1 primarily binds miRNAs and AGO4 binds hc-siRNAs (Zilberman et al. 2003; Baumberger and Baulcombe 2005; Qi et al. 2006; Havecker et al. 2010).

MiRNAs and siRNAs are further distinguished by their dependency on DNA-dependent RNA polymerases for their production. MiRNAs are typically transcribed by Pol II whereas hc-siRNA sequences are transcribed by Pol IV. The Pol IV transcripts serve as templates for RDR2 to generate dsRNAs that are processed by DCL3 into 24-nt hc-siRNAs and then loaded onto AGO4-containing complexes (Pontier et al. 2005; Mosher et al. 2008; Haag and Pikaard 2011). The AGO4-bound hc-siRNAs are recruited by nascent Pol V transcripts which then guide RNA-directed DNA methylation (RdDM). Along with this, Pol IV and Pol V have distinct roles in the RdDM pathway, Pol IV being required for transcription of precursor RNAs from heterochromatic loci, and Pol V transcripts being required for siRNA targeting to the RdDM-affected loci.

During the last years the number of plant miRNAs registered in miRBase (http://microrna.sanger.ac.uk) has dramatically increased, this expansion being largely a benefit of the adoption of next-generation high-throughput sequencing technology. Although the early criteria for miRNA annotation based on expression and biogenesis still provide a broadly accepted standard for miRNA annotation (Ambros et al. 2003), additional criteria were proposed to strengthen plant miRNA annotations (i.e identification of miRNA and miRNA* sequences and DCL dependency for miRNA accumulation) (Meyers et al. 2008). However, some released miRNAs might still be incorrectly annotated and not assessed with sufficient stringent criteria prior to their addition to the database.

Plant miRNAs are known to play important roles in a wide range of developmental processes (Palatnik et al. 2003; Mallory et al. 2004). MiRNAs also regulate the miRNA pathway itself (Mallory and Vaucheret 2006). There is also increasing evidence that the modulation of miRNA levels plays an important role in reprogramming plant responses to abiotic stress, including drought, cold, salinity, and nutrient deficiency (Chiou et al. 2006; Jagadeeswaran et al. 2009). In Arabidopsis,

miR396 acts as a developmental regulator in the reprogramming of root cells during cyst nematode infection (Hewezi et al. 2012). New insight into miRNA function was gained with the discovery that distinct miRNAs target genes involved in plant immune responses to pathogen infection (Katiyar-Agarwal and Jin 2010; Li et al. 2012; Shivaprasad et al. 2012; Jang-Kyun Seo et al. 2013; Pumplin and Voinnet 2013; Staiger et al. 2013).

Traditionally, studies on plant immunity focused on the transcriptional regulation of protein-coding genes. Along with this, host-encoded receptors recognize pathogen-associated molecular patterns (PAMPs, previously known as elicitors). This recognition elicits the PAMP-triggered immunity (PTI), or basal disease resistance, a process in which regulation of immune-response genes occurs through the coordinated regulation of hormone signals (Jones and Dangl 2006). Some pathogens, in turn, deliver effector proteins into the host cell that interfere PTI functions and allow successful infection. As another layer of defense, plants have developed the ability to recognize such microbial effectors by additional receptors (resistance proteins, R) to activate the effector-triggered immunity (ETI). In this context, it is becoming apparent that small RNAs can modulate host gene expression in both PTI and ETI (Katiyar-Agarwal and Jin 2010; Li et al. 2012; Shivaprasad et al. 2012; Jang-Kyun Seo et al. 2013; Pumplin and Voinnet 2013; Staiger et al. 2013). The important role of the Arabidopsis miR393 in antibacterial resistance is well documented (Navarro et al. 2006). Thus, infection with the bacterial pathogen *P. syringae* as well as treatment with the bacterial elicitor flagelin (flg22, a well-studied PAMP from *P. syringae* flagellin) induces miR393 accumulation which, in turn, silences the expression of the TIR1 (TRANSPORT INHIBITOR RESPONSE1) auxin receptor. Repression of auxin signaling then contributes to bacterial resistance (Navarro et al. 2006). MiRNAs that guide the cleavage of transcripts corresponding to *R* genes and trigger production of phased secondary siRNAs have been characterized in *Solanaceae* and *Leguminosae* species in relation to antiviral and antibacterial resistance (Li et al. 2012; Shivaprasad et al. 2012). In rice, we recently described a miRNA, miR7695 that positively regulates resistance to infection by the rice blast fungus *Magnaporthe oryzae* (Campo et al. 2013). Indeed, together with the knowledge of pathogen-responsive genes (i.e. plant antifungal genes), a better knowledge of smRNAs involved in plant immunity will contribute to delineate novel strategies to improve disease resistance in plants.

Most studies on pathogen-regulated smRNAs have been conducted in Arabidopsis plants infected with the bacterial pathogen *Pseudomonas syringae,* or treated with the flagelin-derived elicitor peptide flg22 from *P. syringae*. However, less is known about smRNAs that are regulated during infection with fungal pathogens in plants. To fill this knowledge gap, we conducted a microarray-based search to identify Arabidopsis small RNAs whose expression is affected by treatment with fungal elicitors. Among the elicitor-regulated miRNAs was miR168, known to control AGO1 homeostasis. Both the precursor and mature miR168 were induced by elicitor treatment. Consistent with this, the *MIR168a* promoter is transcriptionally activated by fungal elicitors in transgenic Arabidopsis plants harboring the *promMIR168a::GFP* (green fluorescent protein) fusion gene. Using transgenic plants expressing a *GFP-miR168** sensor construct, we further demonstrated that the elicitor-induced miR168 is active. Microarray analysis also revealed elicitor responsiveness of a small RNA currently annotated as miR415 in miRBase. The status of this sequence has been, however, questioned (http://microrna.sanger.ac.uk). Using Arabidopsis mutants impaired in small RNA biogenesis and function, we show that production of this particular small RNA depends on Pol IV, DCL3 and RDR2 activities. Based on these findings, we conclude that this small RNA is an hc-siRNA, and can no longer be considered a miRNA. Microarray experiments also revealed elicitor-responsiveness of miR823, this miRNA targeting the plant-specific methyltransferase involved in DNA methylation (Chromomehylase 3, CMT3). These findings further support a role for hc-siRNAs, potentially acting in RdDM processes, in the response of Arabidopsis plants to fungal elicitors.

Results

Identification of small RNAs those are responsive to fungal elicitors in Arabidopsis

In this work, we examined alterations in the accumulation of Arabidopsis small RNAs in response to treatment with elicitors obtained from the fungus *Fusarium oxysporum* (Coca and San Segundo 2010). Towards this end, we used a customized microarray containing 2382 probes corresponding to 166 known miRNAs (i.e. small

RNAs mapping at the 5p or 3p arm of the precursor structures annotated in miRBase), 553 candidate miRNAs selected from the literature (Arteaga-Vázquez et al. 2006; Khomicheva et al. 2006; Rajagopalan et al. 2006) and 1096 small RNA sequences derived from massively parallel signature sequencing (MPSS) in Arabidopsis (Meyers et al. 2004a; Meyers et al. 2004b). Probes for known miRNAs from non-plant species (*Caenorhabditis elegans, Drosophila melanogaster*) were also included in the microarray. The complete list of probes represented in the microarray is presented in Table S1.

Total RNA was isolated from Arabidopsis seedlings that had been treated with elicitors obtained from the fungus *F. oxysporum*, and mock-inoculated plants, at different times of treatment (from 5 min to 120 min of treatment). Three independent biological replicates were prepared for each time point and condition. Samples harvested at 5, 30, 60 and 120 min of elicitor treatment were used for microarray experiments. Elicitor-induced alterations we identified by determining the ratio of the hybridization signal intensities between treated and control plants.

Among the small RNAs interrogated in the microarray, 15 miRNAs corresponding to 13 miRNA families, and 81 predicted miRNAs showed elicitor-responsiveness (up- or down-regulation) at one or more time points of elicitor treatment (p value ≤ 0.05; Table 1 and Table S1). Most of these differentially expressed miRNAs showed a dynamic response to fungal elicitors. Only miR168 maintained a constant trend (up-regulation) in its response to fungal elicitors during the entire period of treatment.

Elicitor-regulated miRNAs mainly target transcription factors involved in the control of developmental processes. For instance, miR156 is known to target *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) transcription factors. Two members of the miR156 family, miR156a and miR156h, were found to be sequentially regulated by elicitors (Table 1). Among the group of elicitor-regulated miRNAs were also miR165/166 targeting the PHABULOSA and PHAVOLUTA transcription factors, miR170 targeting SCARECROW-like (SCL), and miR172 targeting APETALA2 transcription factors. Moreover, several miRNAs that regulate auxin homeostasis were identified among the set of elicitor-responsive miRNAs. They were: miR167 targeting two Auxin Response Factors (ARF) genes (*ARF6* and ARF8), miR164 and miR169

(targeting *NAC1* and subunits A of the NF-Y transcription factor complex, respectively). This observation points to a possible miRNA-mediated regulation of auxin signaling by fungal elicitors.

Table 1 Elicitor-responsiveness of known Arabidopsis miRNAs as determined by microarray analysis. MiRNAs whose expression varies in at least one time point of elicitor treatment are listed (for details on the entire set of miRNAs represented in the microarray, see Table S1. The fold change (elicitor-treated *vs.* non-treated plants) for each miRNA is shown. Three biological replicates and three technical replicates for each biological sample were analyzed.

Name	Sequence	Direction of miRNA expression				Target gene	Biological function
		5 min	30 min	60 min	120 min		
miR156a	ugacagaagagagugagcac	n.d.	n.d.	n.d.	-1,79	SPL10 TF (At1g27370) [1]	Development
miR156h	ugacagaagaaagagagcac	-4,13	-2,74	1,41	n.d.	SPL2 (At5g43270) [2]	
miR164a	uggagaagcagggcacgugca	n.d.	1,16	n.d.	n.d.	CUC1/2 TF (At5g53950/At3g15170) [2]	Development. Auxin signaling
miR164c	uggagaagcagggcacgugcg	n.d.	9,5	n.d.	n.d.	NAC080 TF/NAC100 TF (At5g07680/At5g61430) [2]	
miR165a	ucggaccaggcuucauccccc	1,31	n.d.	n.d.	n.d.	PHABULOSA TF/ PHAVOLUTA [TF] (At2g34710/At1g30490) [4]	Development
miR166a	ucggaccaggcuucauucccc	-1,29	n.d.	n.d.	n.d.		
miR168	ucgcuuggugcaggucgggaa	2,88	7,57	2,32	2,1	Argonaute1 (AGO1) (At1g48410) [5]	miRNA functioning. Abiotic stress
miR169d	ugagccaaggaugacuugccg	n.d.	n.d.	n.d.	-2,32	ATHAP2B (At3g05690) [6]	Development. Auxin signaling
miR170	ugauugagccgugucaauauc	n.d.	-1,76	n.d.	n.d.	SCL TF [7]	Development
miR415	aacagagcagaaacagaacau	n.d.	5,25	n.d.	n.d.	questioned miRNA	
miR418	uaaugugaugaugaacugacc	n.d.	n.d.	n.d.	6,63	questioned miRNA	
miR823	ugggguggugaucauauaagau	n.d.	10,7	n.d.	n.d.	Chromomethylase 3 (CMT3) (At1g69770) [8]	Gene silencing
miR833a-5p	uguuuguuguacucggucuagu	4,55	3,1	2,83	-1,28	F-box containing protein (At1g77650) [8]	
miR842	ucauggucagauccgucaucc	n.d.	n.d.	n.d.	3,09	Jacalin lectin (At5g28520) [9]	
miR862-5p	uccaauaggucgagcaugugc	n.d.	-1,73	n.d.	n.d.	Unknown	

[1] (Vaucheret et al. 2004) [2] (Kasschau et al. 2007); [3] (Mallory et al. 2004); [4] (Mallory and Vaucheret 2006); [5] (Jones-Rhoades et al. 2006) ; [6] (Llave et al. 2002); [7] (Jia and Rock 2013). SPL, Squamose promoter binding protein-like; CUC1/2 TF, cup-shaped cotyledon1/2 transcription factor; NAC (NAM, ATAF and CUC) transcription factor; SCL, Scarecrow-like (GRAS TF). -, no change in expression.

Of interest, miR168 was found to be up-regulated by fungal elicitors at all the time points of elicitor treatment examined here. We also noticed a significant increase in miR823 accumulation (FC, 10.66) at 30 min of elicitor treatment. This particular miRNA has been reported to target transcripts of *Chromomethylase 3* (*CMT3*) encoding an enzyme involved in DNA methylation and gene silencing (Rajagopalan et al. 2006). Microarray analysis also showed regulation by fungal elicitors of certain small RNA sequences whose status as a miRNA has been questioned (i.e. miR415 and miR418).

Table 2. Conservation among different plant species of elicitor-responsive miRNAs.

Name	Dicot				Monocot			
	Mt	**Gm**	**Pt**	**St**	**Os**	**Bd**	**Zm**	**Sb**
miR156a	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
miR156h	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
miR164a	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
miR164c	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
miR165a	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
miR166a	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
miR168	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
miR169d	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
miR170	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
miR415	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
miR418	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
miR823	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
miR833a-5p	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
miR842	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
miR862-5p	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)

Mt, *Medicago truncatula*, Gm, *Glycine max*, Pt, *Populus trichocarpa*, St, *Solanum tuberosum*, Os, *Oryza sativa*, Bd, *Brachypodium distachyon*, Zm, *Zea mays*, Sb, *Sorghum bicolor.* + and -, identical and non conserved sequences, respectively).

Next, the conservation of elicitor-responsive miRNAs among different plant species, both dicotyledonous and monocotyledonous species, was investigated. In addition to *Arabidopsis thaliana*, *Medicago truncatula*, *Glycine max*, *Populus trichocarpa*, and *Solanum tuberosum* are the dicot species with the highest number of miRNAs annotated in the miRBase registry. As for monocots, *Oryza sativa*, *Zea mays*, *Sorghum bicolour* and *Brachypodium distachyon* have the highest number of miRNAs

annotated in miRBase. In this work, conserved miRNAs were designated as those having identical sequences with the miRNA sequences annotated in miRBase for all the above mentioned plant species. On this basis, both conserved and non conserved miRNAs were identified among the subset of elicitor-responsive miRNAs (Table 2).

The microarray analysis comparing control non treated and elicitor-treated tissues of Arabidopsis plants also allowed us to examine the accumulation level of small RNA sequences previously identified through MPSS (Meyers et al. 2004a; Meyers et al. 2004b). Up to 98 of those small RNA sequences showed alterations in their accumulation at one or another time point (Table S1). As it was observed for miRNAs, the response of these small RNA sequences to elicitors was highly dynamic, their accumulation being up- and down-regulated during the period of elicitor treatment examined here. Together, microarray analysis revealed an altered expression of a subset of smRNAs, including miRNAs from Arabidopsis, in response to treatment with fungal elicitors, which might be indicative of their possible involvement in the plant response to fungal infection.

Based on the results obtained in microarray experiments, two small RNA sequences were selected for further analysis, namely miR168 and the small RNA sequence annotated as miR415 in miRBase. miR168 was considered of interest due to the important role that this particular miRNA plays in controlling the miRNA machinery, by regulating AGO1 homeostasis. Concerning miR415, the status of this sequence as a miRNA has been questioned. In this context, it was important to investigate whether the elicitor-regulated small RNA sequence named as miR415 in miRBase is a true miRNA from Arabidopsis, or not.

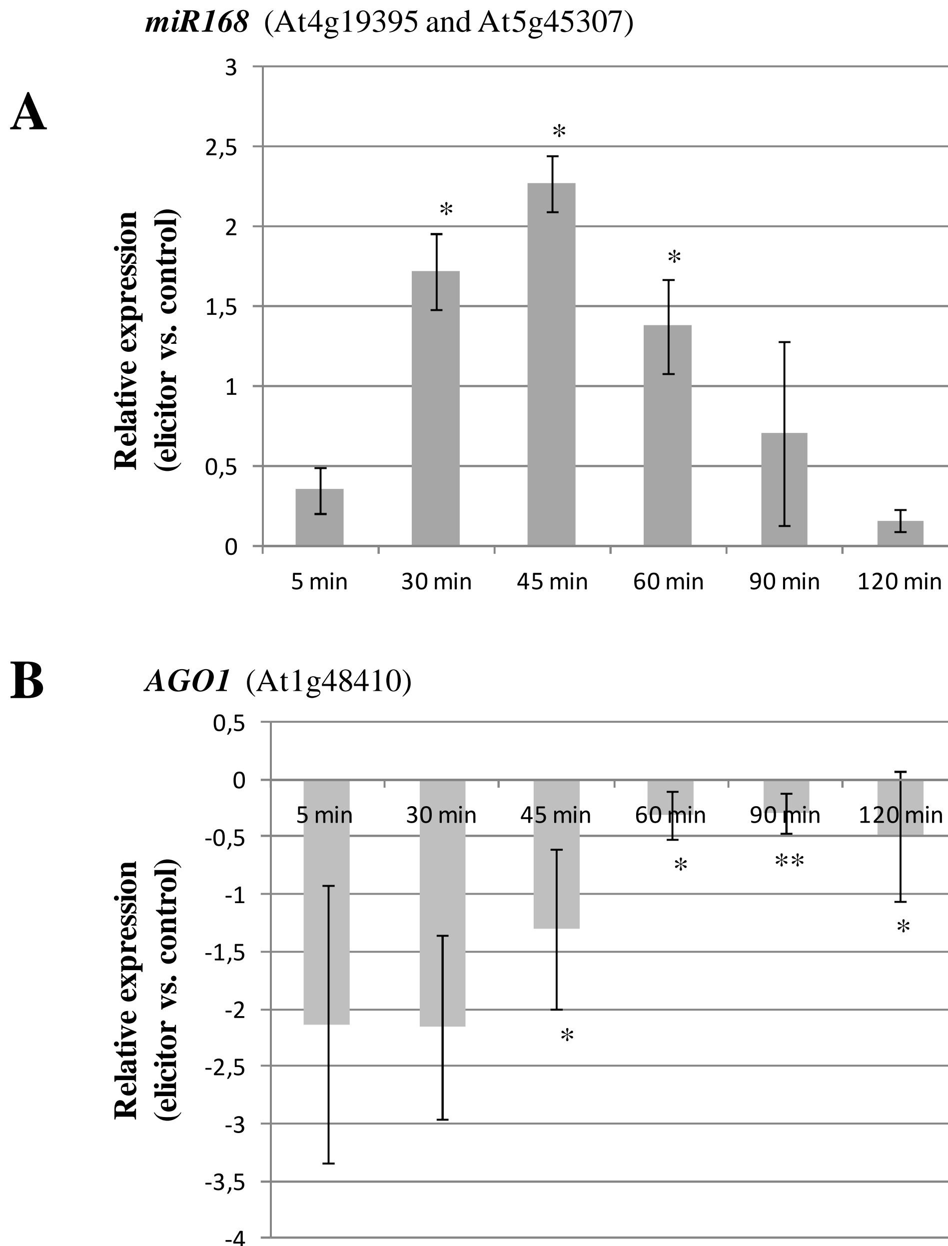

Figure 2. Expression of miR168 and *AGO1* in Arabidopsis plants treated with elicitors from *F. oxysporum*. (A) Northern blot analysis of miR168. RNAs were prepared from Arabidopsis plants treated with fungal elicitors for the indicated periods of time, and from control mock-inoculated plants (RNAs from samples harvested at 5, 30, 60 and 120 min were also used for microarray analysis). Ethidium bromide-stained rRNAs are shown as loading controls. **(B)** qRT-PCR analysis of *AGO1* using *Ubiquitin10* (At5g65080) as the internal control. RNAs samples were the same as in (A). Results shown are from one of three independent experiments that gave similar results. Bars show standard error. c, control plants. e, elicitor-treated plants.

***MIR168* is transcriptionally activated in response to fungal elicitors in Arabidopsis**

Microarray analysis revealed up-regulation of miR168 in response to elicitor treatment. This particular miRNA directs cleavage of *AGO1* mRNA, with AGO1 being the core component of the RISC complex involved in miRNA functioning (Baumberger and Baulcombe 2005). Considering the important role of miR168 in controlling the miRNA machinery, it was of interest to explore the elicitor-responsiveness of both partners, miR168 and *AGO1*. Initially, Northern blot analysis was used to examine mature miR168 accumulation in control and elicitor-treated plants at all time points of elicitor treatment (5, 30, 45, 60, 90 and 120 min). As it is shown in Figure 1a, a marked increase in miR168 accumulation could be observed as early as 5 min after the onset of elicitor treatment. miR168 accumulation remained at higher levels in elicitor-treated plants compared to control plants up to 90 min of elicitor treatment. Thus, results obtained by Northern blot analysis were then consistent with those observed by microarray analysis, further supporting that miR168 is up-regulated by fungal elicitors. On the other hand, *AGO1* expression remained at lower level in elicitor treated tissues compared to control plants at all time points, although these differences gradually decreased during the period of elicitor treatment (Figure 1b).

Collectively, microarray and Northern blot analyses revealed that the mature miR168 accumulates in response to fungal elicitors. Moreover, the elicitor-induced up-regulation of miR168 might account for the observed reduction in *AGO1* transcripts in elicitor treated plants compared to control plants during the period of treatment here assayed.

Next, we investigated the *in vivo* activity of miR168 in tissues (leaves, roots) of control and elicitor-treated Arabidopsis plants. Despite the relevance of *MIR168* in controlling AGO1 homeostasis, little attention has been paid to *MIR168* expression or activity in tissues of Arabidopsis plants other than leaves. Towards this end, a sensor gene was obtained in which the miR168 target sequence was inserted into the 3'-UTR of the *GFP* reporter gene (*GFP-miR168** gene, Figure 2a). Transgenic plants harboring the miR168 sensor gene were generated. MiR168 activity was inferred by monitoring GFP fluorescence in tissues of transgenic Arabidopsis plants (leaves, roots), and in response to treatment with fungal elicitors (the GFP-miR168* sensor is degraded in

cells where miR168 is present). Transgenic plants expressing the *GFP* gene devoid of the miR168 target sequence were produced and used as controls. As expected, GFP fluorescence was visualized in roots and leaves of control *GFP*-Arabidopsis plants (Figure 2b).

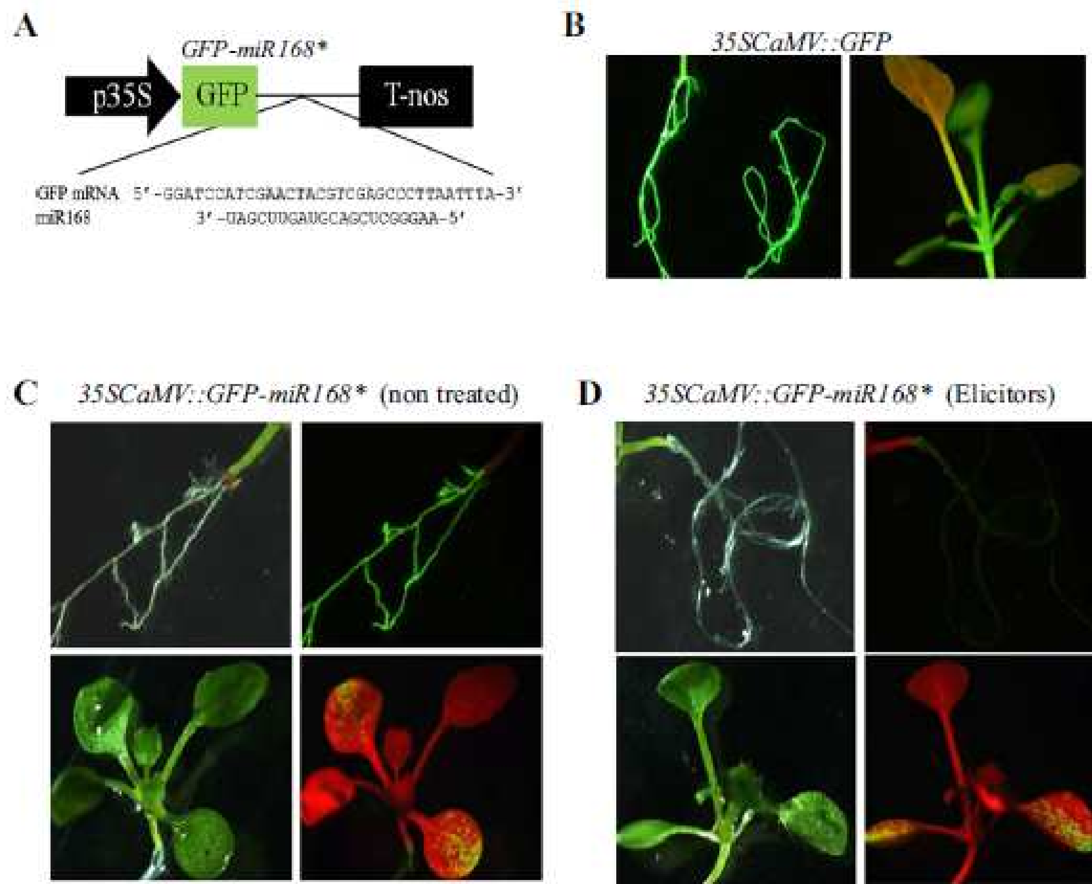

Figure 3. MiR168 activity in Arabidopsis plants revealed by GFP fluorescence patterns tissues of *GFP*-miR168* sensor plants. (A) Schematic representation of the miR168 sensor construct containing the *GFP* mRNA with a site complementary to miR168 (*GFP*-miR168*). (B) Plants constitutively expressing the *GFP* gene. Ten day-old plants were treated for 30 min with elicitors obtained from the fungus *F. oxysporum*. Water was used as mock control. Results obtained in elicitor-treated *GFP*-Arabidopsis plants are presented (similar patterns were observed in non treated *GFP*-Arabidopsis plants). Bright-field (left) and GFP fluorescence (right) images are shown. (C) Analysis of miR168 activity in control, non treated *GFP-miR168** plants (*sde1* background). (D) miR168 activity in elicitor-treated *GFP*-miR168* Arabidopsis plants. No GFP fluorescence was evident in roots of non treated plants due to miR168 guided silencing.

Under control conditions, *GFP* expression was readily detectable in roots of *GFP-mir168** plants whereas only some fluorescent spots were visualized in their leaves, mainly at the distal areas (Figure 2c). Then, we compared the GFP patterns in tissues of control and elicitor-treated *GFP-miR168** Arabidopsis plants. Remarkably, treatment

with fungal elicitors resulted in loss of GFP fluorescence in roots of *GFP*-miR168* plants suggesting miR168 activity in these tissues (Figure 2d, left panels). By contrast, differences in GFP fluorescence pattern between control and elicitor-treated leaves of *miR168-GFP* plants were not evident (Figure 2d, right panels). These findings support that elicitor treatment results in enhanced activity of miR168, mainly in roots of Arabidopsis plants. The elicitor-induced activity that occurs in roots of sensor plants might well reflect an increase in mature miR168 in these tissues, which would account for the higher level of miR168 accumulation that is observed in elicitor-treated plants compared to non treated plants by Northern blot analysis (see Figure 1a).

In Arabidopsis, the miR168 family comprises two members with identical mature miRNA sequences, miR168a and miR168b. However, neither microarray or Northern blot analyses, nor the GFP sensor system, provide information on the expression of the individual members of this miRNA family. To investigate the elicitor-responsiveness of each individual family member, we examined the expression of their corresponding precursors (pre-miR168a and pre-miR168b) by qRT-PCR. This study revealed an important increase on the accumulation of both miR168 precursors at 30 min of elicitor treatment which progressively returned to normal values (Figure 3a). These findings support a transcriptional activation of the two miR168 family members, miR168a and miR168b, in response to treatment with fungal elicitors.

It is generally assumed that most *MIR* genes are transcribed by RNA polymerase II and that their upstream regulatory regions contain canonical regulatory elements, i.e. *cis*-elements that are known to regulate transcription of protein-coding genes (Xie et al. 2005a; Megraw et al. 2006). Previous studies demonstrated that the *MIR168a* promoter contains numerous abscisic acid-responsive elements (ABREs). Along with this, mature miR168 accumulated in response to abiotic stress and ABA treatment in Arabidopsis (Li et al. 2012).

In this work, we scanned the *MIR168* promoter region for the presence of known *cis*-elements related to biotic stress and defense-related hormones. The sequence upstream of the precursor structure for either pre-miR168a or pre-miR168b was extracted from the NCBI database and the transcription start site (TSS) was identified by using the transcription start site identification program for plants (http:// http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=prom

oter). *cis*-acting elements present in the 2Kb DNA region upstream of the TSS were searched using the PLACE database (http://www.dna.affrc.go.jp/PLACE) (Higo et al. 1999). Interestingly, the *MIR168a* and *MIR168b* promoters were found to contain several pathogen-responsive *cis*-elements (Figure 3b; Table S2). Among them, we identified the elicitor responsive element (ERE; TTGACC) as well as several W-boxes,

including the WRKY71 and W-boxNPR1 elements. The ERE regulatory element has been shown to direct pathogen- and elicitor-responsive expression in many stress-related genes (Raventos et al. 1995; Rushton and Somssich 1998). W-boxes are the binding sites for SA-induced WRKY transcription factors that are also found in the promoter of the *NPR1* (nonexpressor of *PR* genes1) gene, a key regulator of salicylic acid (SA)-mediated defense responses in Arabidopsis (Cao et al. 1997). The SEBF binding site was also identified in the *MIR168* promoter. This regulatory element was initially characterized in the pathogen and elicitor inducible potato pathogenesis-related gene *PR-10a* gene, and later on in the promoter of several other defense-related genes, including *PR* genes. Additional regulatory elements identified in the *MIR168* promoter are associated with defense-related hormones such as ethylene (ET) and methyl jasmonic acid (MeJA) (Figure 3b and Table S2). To note, several auxin-responsive elements (AuxRE), such as the Auxin Response Factor (ARF) binding site and the Small Auxin-Up RNA (SAUR) binding site are present in the *MIR168* promoter. Furthermore, POBO analysis (http://ekhidna.biocenter.helsinki.fi/poxo/pobo/pobo) allowed us to compare the frequency of each motif identified in the miR168 promoter with the frequency of the same motif in the promoter regions of all known genes from Arabidopsis (Kankainen and Holm 2004). Excluding the WboxNPR1, all the pathogen-related *cis*-motifs were significantly enriched in the *MIR168* promoter relative to the Arabidopsis background set (t-test, p-value < 0.0001) (Figure S1). The SURE, LeCp binding site and GARE elements were also found to be significantly enriched in this promoter. Thus, the observed elicitor-induced accumulation of miR168 is consistent with the presence of pathogen/elicitor responsive elements in the *MIR168* promoter.

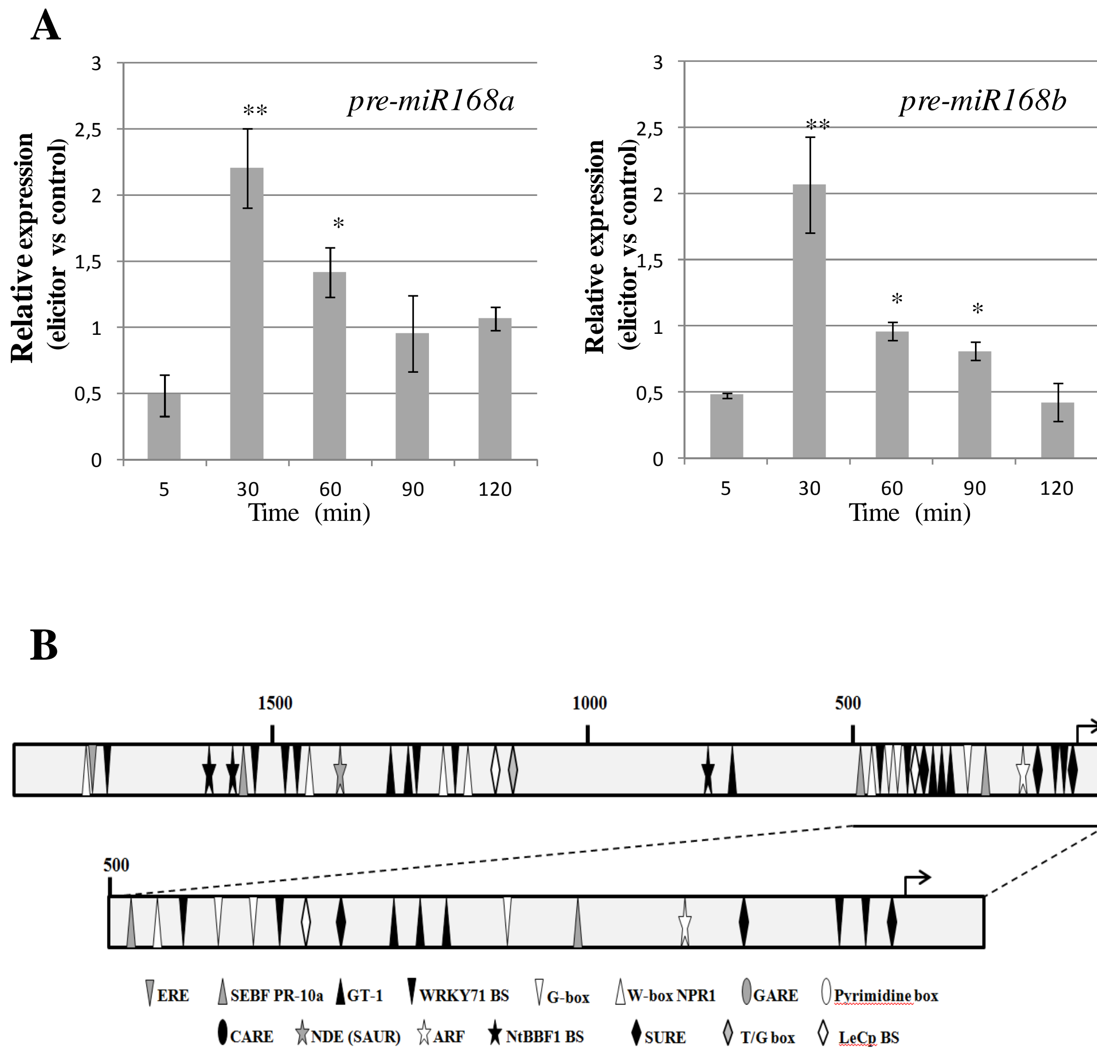

Figure 4. Expression of miR168 precursors and structural features of the *MIR168* promoter. **(A)** qRT-PCR analysis of pre-miR168a (left panel) and pre-miR168b (right panel) expression in response to elicitor treatment. The relative expression level in comparison to the corresponding non treated controls is given for each time point (elicitor *vs.* control non treated plants). Error bars represent the mean ± SD of two biological replicates and three technical replicates for each biological replicate. All values were normalized against *Ubiquitin*. **(B)** Structural features of the *MIR168a* promoter from Arabidopsis. The location of known *cis*-acting elements is shown (for details on *cis*-elements, see Table S2).

To further explore the regulatory mechanism of miR168 in the elicitor response, a functional analysis of the *MIR168* promoter was performed, focusing on the *MIR168a* promoter. For this, the *MIR168a* promoter region was fused to the *GFP* gene and the resulting construct (*pMIR168a::GFP*, Figure 4a) was used to transform Arabidopsis plants. As control, transgenic Arabidopsis plants expressing the *GFP* gene under the control of the *35SCaMV* were generated. As expected GFP fluorescence was observed

in all tissues of *35SCaMV::GFP* plants (Figure 4b). *GFP* expression could not be detected in tissues of control non treated *pMIR168a::GFP* plants (Figure 4c). When comparing these results with those obtained in *GFP-miR168** sensor plants also grown under control conditions (e.g. non treated plants, see Figure 2c) it is concluded that neither *MIR168a* promoter activity nor miR168 activity occurs in non treated roots of *GFP-miR168** plants). An apparent discrepancy occurs in leaves of non treated plants as miR168 activity (see Figure 2c), but not miR168a promoter activity (Figure 4c), is observed in these tissues pointing to the existence of additional regulatory mechanisms controlling *MIR168* expression and functioning under normal conditions. In this respect, a transcriptional/translational interlocked feedback loop governing expression of the miR168/AGO1 pair is well documented in Arabidopsis. Indeed, the level of AGO1 mRNA is regulated by both the miRNA miR168 and by siRNAs generated from the AGO1 mRNA after miR168-mediated cleavage (Mallory et al. 2004; Vaucheret et al. 2004; Mallory and Vaucheret 2006). Clearly, further studies are needed to clarify this aspect.

Of interest, GFP was readily detectable in roots of *pMIR168a::GFP* plants that have been treated with fungal elicitors (Figure 4d, left panels) indicating miR168 promoter responsiveness to fungal elicitors, at least in this organ. This finding is consistent with results obtained in *GFP-miR168** sensor plants, where the elicitor-induced miR168 accumulation resulted in loss of GFP fluorescence (see Figure 2d). The *MIR168* promoter was also found to be activated in elicitor-treated leaves of *pMIR168::GFP* plants (Figure 4d, right panels). Thus, functional analysis of the *MIR168a* promoter in transgenic Arabidopsis plants confirmed its elicitor-responsiveness while revealing a strong activity of this promoter in roots.

Collectively, results obtained by Northern blot and miR168 precursor expression analyses, in combination with those obtained in *GFP-miR168** sensor plants and *pMIR168::GFP* plants, support that *MIR168* is transcriptionally activated by fungal elicitors to produce active miR168 in Arabidopsis plants.

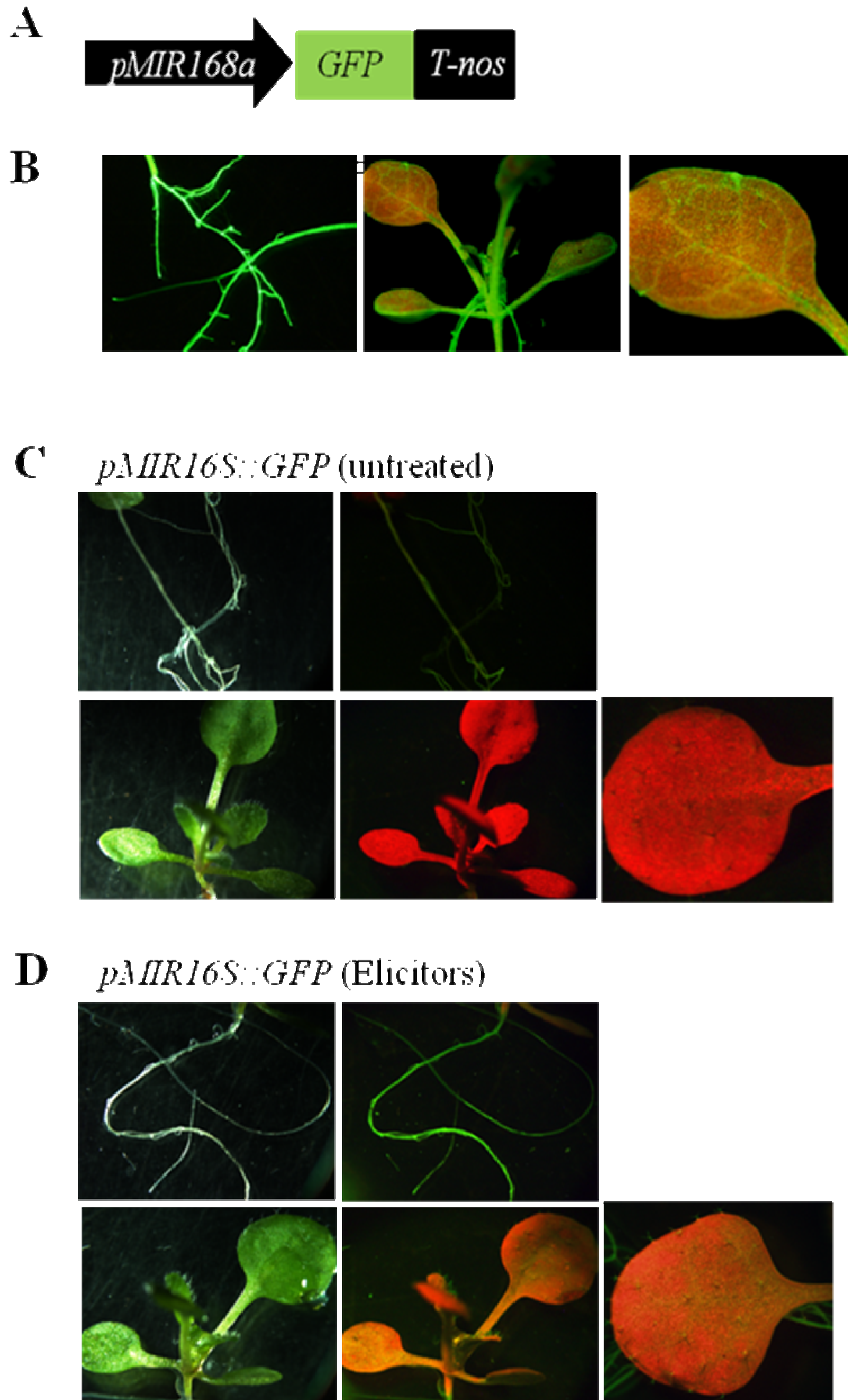

Figure 5 Functional analysis of the *MIR168a* promoter in transgenic Arabidopsis. (A) Schematic diagram of the *MIR168a* promoter construct. (B) Arabidopsis plants constitutively expressing *GFP*. Ten day-old plants were treated with fungal elicitors for 30 min. Water was used as mock control. (C) Control non treated *pMIR168::GFP* plants. (D) Elicitor-treated *pMIR168::GFP* plants.

siRNA415, an elicitor-inducible hc-siRNA from Arabidopsis

The current release of miRBase contains a sequence annotated as miR415 with two entries, one in *A. thaliana* (aacagagcagaaacagaacau) and another in rice (aacagaacagaagcagagcag). This small RNA sequence was detected by Northern blot analysis in Arabidopsis flowers (Vaucheret et al. 2004; Wang et al. 2004). However, the status of this sequence as a miRNA has been questioned due to the lack of conservation in genomes other than Arabidopsis and rice, its moderately poor precursor hairpin structure, and the lack of identified targets.

Our microarray analysis revealed a significant increase in the accumulation of this particular small RNA at 30 min of elicitor treatment (FC 5.25; Table 1). Differences in the accumulation of this small RNA sequence between elicitor-treated and control non treated tissues were also distinguished by small RNA Northern blot (Figure S2). Intriguingly, Northern blot analysis revealed that this small RNA was 24 nucleotides in length, thus, longer than the annotated sequence in miRBase (21-nt in length). To further investigate the nature of this small RNA sequence, we monitored its accumulation in Arabidopsis mutants impaired in small RNA biogenesis.

Initially, we examined mutants with defects in each *DCL* gene, namely *dcl1-9* (a viable hypomorph), *dcl2-1*, *dcl3-1*, and *dcl4-2*, as well as mutants with defects in a *RDR* gene for which a function has been established, namely *rdr1-1*, *rdr2-1* and *rdr6-15* mutants (Jacobsen et al. 1999; Xie et al. 2004; Allen et al. 2005; Xie et al. 2005a). Except for *dcl1-9* (Ler background), all the *dcl* and *rdr* mutants were in the Col0 genetic background. The small RNA under study was detected in wild-type plants of the two accessions, its level of accumulation being slightly lower in Ler plants than in Col0 plants (Figure 5a, upper panel). Interestingly, the *dcl3* mutation abolished the accumulation of this small RNA, whereas there were no significant changes on its accumulation in *dcl1*, *dcl2*, and *dcl4* mutants (Col 0 background) (Figure 5a, upper panel). As expected, accumulation of miR171, a canonical miRNA from Arabidopsis, was reduced in the *dcl1* mutant, but not in any of the other *dcl* mutants (Figure 5a).

As previously mentioned, RDR dependencies are characteristic of plant siRNAs. When examining accumulation of the small RNA in *rdr* mutants, its accumulation was found to be compromised in the *rdr2* mutant, but not in the *rdr1* and *rdr6* mutants (Figure 5a).

Evidence exists that 24-nt hc-siRNAs function in RNA-directed DNA methylation (RdDM) in the chromatin silencing pathway. In this work we examined the accumulation of the 24-nt small RNA in Pol IV and Pol V mutants. As previously mentioned, RdDM requires the concerted action of Pol IV and Pol V, the Pol IV being required for hc-siRNA biogenesis (through the RDR2/DCL3 pathway), and Pol V being responsible of targeting siRNAs to loci controlled by RdDM. Pol IV and Pol V each has a unique largest subunit (NRPD1 and NRPE1, respectively) and share the second largest subunit (NRPD2/NRPE2) (Haag and Pikaard 2011). As it is shown in Figure 5b, production of the 24-nt small RNA is compromised in the *nrpd2* mutant (second largest subunit, common to Pol IV and Pol V), its accumulation being also significantly reduced in the *nrpe1* mutant (largest subunit of Pol V). Together, results obtained in the analysis of *dcl, rdr,* and *pol* mutants were consistent with the interpretation that the 24-nt small RNA under study is produced by the PolIV/RDR2/DCL3 pathway, a typical feature of hc-siRNAs. Finally, hc-siRNAs are known to function in association with AGO4 (Zilberman et al. 2003; Matzke et al. 2009). As it is shown in Figure 5b, accumulation of this small RNA was clearly reduced in the *ago4-2* mutant (Col 0 background), this observation supporting that this small RNA associates with AGO4.

As controls, we examined the accumulation of the 24 nt hc-siRNA originating from 5S rDNA (siRNA1003) in the panel of Arabidopsis mutants. Previous studies demonstrated that production of siRNA1003 is Pol IV- and Pol V-dependent (Herr et al. 2005; Onodera et al. 2005; Pontier et al. 2005) and that siRNA1003 accumulation is significantly reduced, or abolished, in *dcl3-1* and *rdr2-1* mutants (Xie et al. 2005a; Xie et al. 2005b). Consistent with previously reported results, siRNA1003 was reduced to undetectable levels in *dcl3*, *rdr2*. siRNA1003 production was also impaired in the *nrpd2* (second largest subunit of Pol IV and Pol V) mutant, while showing an important reduction in the *nrpe1* mutant (Pol V largest subunit) compared to wild type Col0 plants (Figure 5b). As expected, siRNA1003 accumulation was not affected in *dcl1-9*, *dcl2-1*, *dcl4-2*, *rdr1-1* and *rdr6-15* mutants (Figure 5a). Thus, siRNA1003 and the siRNA under study exhibit the same genetic requirements of their production, namely Pol IV, RDR2 and DCL3.

Taken together, analysis of Arabidopsis mutants affected in biogenesis and functioning of small RNAs revealed that mutations in any of the genes in the Pol IV/RDR2/DCL3 pathway affect the accumulation of this small RNA species. This piece

of evidence strongly supports that the 24-nt small RNA investigated here is indeed a hc-siRNA and not a miRNA. This sequence may therefore be removed in subsequent data releases from miRBase. We have named this small RNA siRNA415.

Finally, we examined whether siRNA415 occurs in plant species other than Arabidopsis. For this, we examined its accumulation in several monocotyledonous (*Zea mays*, *Sorghum bicolour*, *Oryza sativa*), and dicotyledonous (*Cucumis melo*, *Nicotiana tabacum*, *Solanum lycopersicum*) species. As it is shown in Figure 5c, siRNA415 was found to accumulate at different levels in the various plant species examined here.

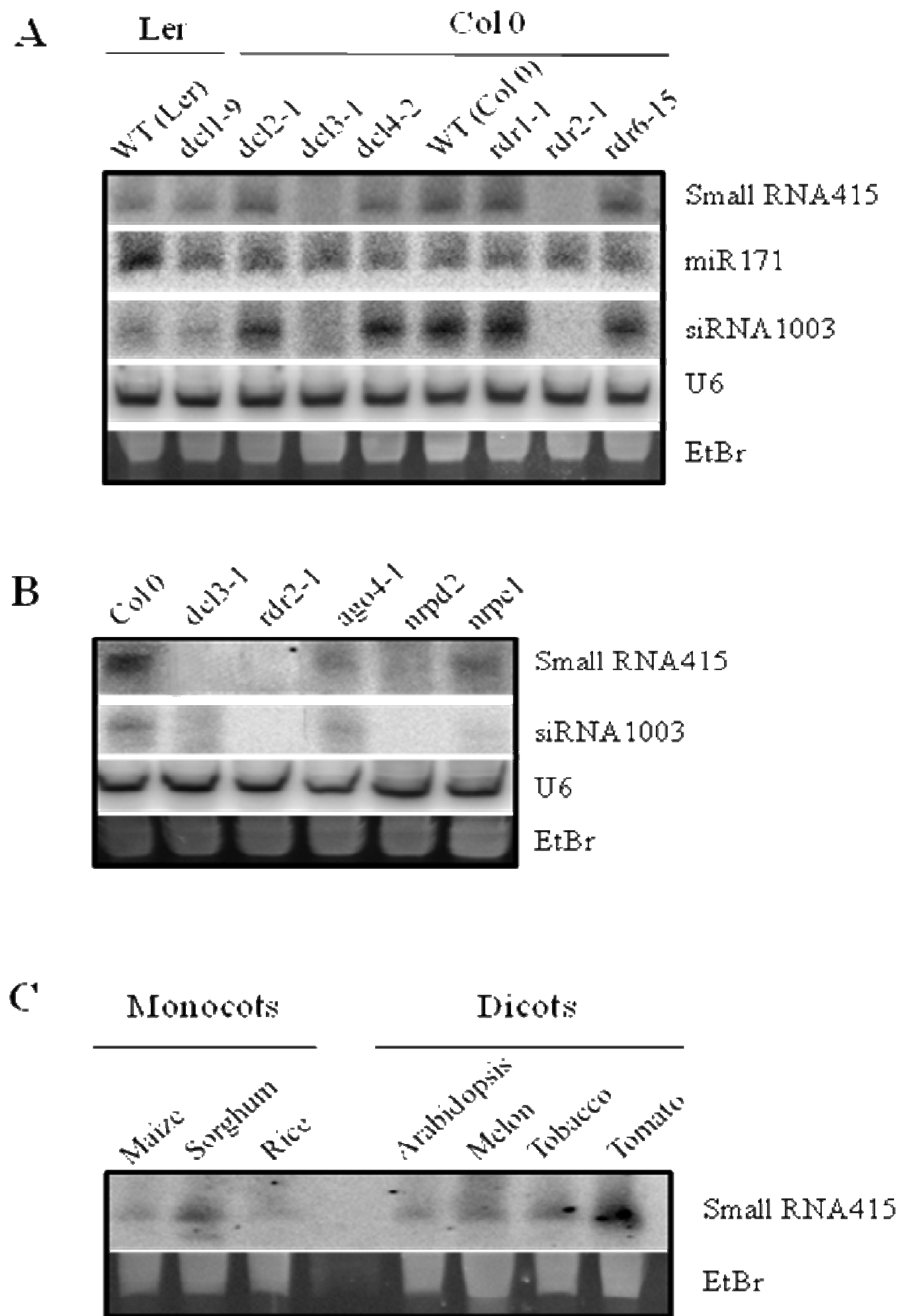

Figure 6. Genetic requirements for generation of the 24-nt hc-siRNA415. (A) Analysis of mutants impaired in small RNA biogenesis, *dcl* and *rdr* mutants. The same blot was successively hybridized, stripped, and re-hybridized to oligonucleotide probes corresponding to the complementary sequence of the indicated small RNAs. RNA blots were also probed with the U6 probe for loading control. (B) Analysis of *ago4*, *nrpd2* (common to Pol IV and Pol V) and *nrpe1* (Pol V) mutants. (C) Small RNA blot analysis of the hc-siRNA415 in different plant species.

Discussion

In this work, we identified a group of small RNAs that are regulated by fungal elicitors pointing to a possible role for these particular smRNAs in PAMP-triggered immunity in Arabidopsis plants. An important number of the elicitor-regulated miRNAs identified in this study are known to control the expression of transcription factors functioning in developmental regulation. This observation further supports a link between plant development and pathogen resistance in Arabidopsis while providing evidence that these miRNAs might be regulated by both developmental cues and biotic stress conditions. Because miRNAs provide quantitative regulation of target gene expression, rather than on-of regulations, the group of elicitor-regulated smRNAs identified in this work might contribute to fine-tune gene expression in reprogramming developmental programs, this process being part of the adaptive strategy of plants to pathogen infection.

Among the set of elicitor-regulated miRNAs here identified, there were several miRNAs controlling genes involved in hormone signaling, namely auxin signaling. In addition to their role in plant development, auxins play an important role in hormone crosstalk during the plant's stress response (Park et al. 2007). Antagonism between auxin and SA, a major regulator of plant defenses, has long been demonstrated (Wang et al. 2004). Auxins are also known to regulate the expression of plant defense genes (Ding et al. 2008). In other studies, repression of the auxin response pathway was found to increase Arabidopsis susceptibility to necrotrophic fungi (Llorente et al. 2008). The repression of auxin signaling in Arabidopsis enhances resistance to bacterial infection in Arabidopsis, a process that is mediated by miR393 (Navarro et al. 2006). Under this scenario, it can be postulated that recognition of fungal elicitors would trigger alterations in the expression of distinct miRNAs which are responsible of reprogramming host developmental processes, including auxin-regulated processes. This perturbation, in turn, might contribute to regulation of defense responses either directly or indirectly through cross-talk between auxin and defense-related hormones.

In our microarray analysis, however, no changes were observed in the expression of the bacteria-responsive miR160 and miR390, also known to control genes involved in auxin signaling pathway. This observation might be indicative of a differential

regulation of these miRNAs during infection by either fungal pathogens or bacterial pathogens. Alternatively, the expression of miR160 and miR390 (and perhaps miR393 which was not represented in the microarray) might be regulated at time points of elicitor treatment not assayed in this work. Clearly, regulation of multiple miRNAs exhibiting regulatory links with auxin signaling during PTI responses might provide a fine tune regulation of gene expression in controlling resistance to different types of plant pathogens.

On the other hand, our study revealed that miR168, a miRNA that is deeply conserved among plant species, is transcriptionally regulated by fungal elicitors. Several lines of evidence support this conclusion. Firstly, we demonstrated that mature miR168 rapidly accumulates in response to elicitor treatment. Secondly, expression of miR168 precursors is also up-regulated upon elicitor treatment. Thirdly, functional analysis of the *MIR168a* promoter in transgenic Arabidopsis further supports that *MIR168* is regulated at the transcriptional level. The observed transcriptional activation of *MIR168* is consistent with the presence of various pathogen- and elicitor-responsive *cis*-elements in the *MIR168* promoter. Finally, by using GFP-miR168* sensor plants we demonstrated that the elicitor-induced miR168 is active.

It is well established that miR168 plays a critical role in miRNA functioning by controlling AGO1 homeostasis. Thus, AGO1 homeostasis entails coexpression of *MIR168* and *AGO1* and preferential stabilization of miR168 by AGO1 (Vaucheret 2006). In this work, we show that the elicitor-induced accumulation of miR168 leads to a consistent decline on the accumulation of *AGO1* transcripts in elicitor-treated tissues relative to control tissues (i.e. differences between elicitor and control plants progressively diminished during elicitor treatment). On the basis of these data, we can hypothesize that a transcriptional regulation of *MIR168* might be responsible for the control of *AGO1* accumulation early during elicitor treatment. If so, the transcriptional regulation of *MIR168* would represent another layer of control of the refined regulatory system that controls miR168 levels and AGO1 levels. Presumably, an elicitor-regulated adjustment of miR168 levels might contribute to the maintenance of the appropriate levels of AGO1, and accordingly of miRNA functioning, during the plant defense response to pathogen infection.

In the literature there are several reports on the involvement of AGO1 in plant antiviral defense (Várallyay et al. 2010). AGO1 also contribute to flg22-induced disease resistance in Arabidopsis plants (Li et al. 2010). Very recently, Shen et al. (Shen et al. 2014) described a decrease in miR168 accumulation with a simultaneous increase in AGO1 transcript abundance in roots of oilseed rape (*Brassica napus*) infected with the soil-borne pathogenic fungus *Verticillium longisporum* (at 6 days after inoculation). Furthermore, Weiberg et al. (2013) demonstrated that some small RNAs from *Botrytis cinerea* hijack the host RNAi machinery by binding to AGO1 and selectively silencing host immunity responses. Together, these pieces of evidence strongly support that miR168/AGO1 modulation might play an important role in shaping host responses to pathogen infection, including infection by fungal pathogens. In other studies, miR168 was reported to be induced by abiotic stresses (drought, salinity, cold) (Li et al. 2012). Then, it is noteworthy that miR168 is regulated by both biotic and abiotic stress conditions, these findings highlighting the importance of miRNA functioning in plant adaptive processes to environmental stress. Clearly, a fine-tuned adjustment of miR168 and AGO1 levels would provide a flexible system for the control of processes that are critical to ensure plant survival under adverse environmental conditions.

On the other hand, our microarray analysis of Arabidopsis small RNAs allowed us to identify a small RNA (24-nt in length) that transiently accumulates in response to fungal elicitors. Most importantly, analysis of mutants impaired in the small RNA biogenesis pathways revealed that production of this 24-nt siRNA is dependent on the DCL3/RDR2/Pol IV pathway, its accumulation being also reduced by mutations in Pol V and AGO4. As previously mentioned, biogenesis of hc-siRNAs begins with the transcription by RNA Pol IV, which is then followed by RDR2-catalyzed synthesis of dsRNA. Then, si-RNAs (24-nt) are processed from the dsRNAs by DCL3, and loaded into AGO4-containing RISCs. Besides, the function of the AGO4-assembled hc-siRNAs requires the production of a scaffold transcript by Pol V which recruits the AGO4-bound hc-siRNAs. Based on the results obtained in this work, it is concluded that siRNA415 is a *bona fide* hc-siRNA that is dependent on Pol IV/RDR2/DCL3/AGO4 for its biogenesis and function. This small RNA sequence was incorrectly annotated as a miRNA and, accordingly, this entry may be removed from future database releases.

In plants, hc-siRNAs are involved in transcriptional gene silencing by guiding DNA methylation at target genomic loci through RNA-directed DNA methylation (RdDM) (Chan et al. 2004; Matzke et al. 2009). A major function of hc-siRNAs is to maintain genome integrity by silencing transposable elements (Zhong et al. 2012). Some reports indicate that hc-siRNAs also control the expression of protein-coding genes (i.e. the FLOWERING LOCUS C, FLC gene) (Liu et al. 2004). There is then the possibility that siRNA415 might guide DNA methylation at still unknown genomic loci. Further studies are needed to assess whether siRNA415 guides methylation in Arabidopsis plants. Finally, as this small RNA was identified in plant species other than Arabidopsis, both monocotyledonous and dicotyledonous species, there is also the possibility that functioning of this small RNA is not restricted to Arabidopsis.

The scaffolding model for the function of AGO4-associated hc-siRNAs in RdDM implies recruiting DNA methyltransferase enzymes by transcripts being transcribed by Pol V. In this context, DNA methylation in RdDM is maintained by the overlapping functions of Methyltransferase 1 (MET1) and CMT3 in Arabidopsis. Of interest, microarray analysis revealed elicitor-regulation of miR823, this particular miRNA targeting *CMT3* transcripts. The *CMT3* gene encodes CHROMOMETHYLASE3, a protein involved in RdDM. The finding that miR823 is affected by fungal elicitors raises interesting questions concerning the mechanisms by which this particular miRNA might exert its regulatory role in plant immunity by controlling CMT3 expression and RdDM.

Concerning the involvement of RdDM in mediating regulation of plant immune responses, this issue has only recently come to light (Dowen et al. 2012). Recent reports have shown that DNA methylation is part of the Arabidopsis immune response, i.e. by priming transcriptional activation of some defense genes during antibacterial resistance (Dowen et al. 2012; Yu et al. 2013). A model was proposed to explain regulation of *A. thaliana* immune system by DNA demethylation whereby DNA methylation imparts persistent control over some defense genes during non stressful conditions, but can change dynamically to alter gene expression in response to biotic stress (Dowen et al. 2012). The contribution of components of the RdDM pathway, such as AGO4 and RNA Pol V, in plant immunity is also documented (Agorio and Vera 2007; López et al. 2011). Because RdMD can be rapidly reversed by biotic stress, it was proposed that

dampening defense gene expression through active RdDM would provide an effective mode of regulation of host defense responses in plants (Pumplin and Voinnet 2013).

In summary, results here presented will help in understanding the contribution of small RNAs, both miRNAs and siRNAs, in the Arabidopsis response to pathogen infection while providing new opportunities to elucidate the molecular events controlling plant disease resistance.

Conclusion

In this study microarray analysis was used to identify small RNAs, miRNAs and siRNAs whose accumulation is regulated by treatment with fungal elicitors in Arabidopsis. Among the elicitor-regulated miRNAs was miR168, this miRNA controlling the level of *AGO1* transcripts. We demonstrated that *MIR168* is transcriptionally regulated by fungal elicitors. These finding suggest that miR168 contributes to the maintenance of the appropriate levels of AGO1, and hence miRNA functioning, in the response of Arabidopsis plants to fungal elicitors. Additionally, we identified an elicitor-regulated hc-siRNA (named siRNA415) which was incorrectly annotated as a miRNA. These results, toghether with the observation that miR823 (targeting CHROMOMETHYLASE3 transcripts) further support a function of RNA-directed DNA methylation processes in mediating plant immune responses. This work represents an effort to identify relevant small RNAs regulating the Arabidopsis response to pathogens that may have relevance to study other pathosystems.

Materials and Methods

Plant material and treatment with fungal elicitors

Columbia 0 (Col 0) accession of *A. thaliana* was used for this study. Plants were grown on MS0 medium at 22 ±2°C during 15 days under neutral day conditions (12h light/12h dark). Fungal elicitors were prepared from the fungus *Fusarium oxysporum* (strain 247) as previously described (Coca and San Segundo 2010). Arabidopsis seedlings were treated with a suspension of fungal elicitors at a final concentration of 300 µg/ml (in sterile water) or mock-inoculated. Plant material was harvested at

different time points of elicitor treatment, ground in liquid nitrogen and stored at -80°C. Three independent biological replicates (each one representing approximately 150 plants) were analyzed. The following homozygous Arabidopsis mutants were used in this work: *dcl1-9*, *dcl2-1, dcl3-1, dcl4-2*, *rdr1-1, rdr2-1, rdr6-15, ago4-1, nrpd2* (also named *ocp1*), *nrpe1* (Jacobsen et al. 1999; Zilberman et al. 2003; Pontier et al. 2005; López et al. 2011). The wild-type backgrounds for these mutants were *Landsberg erecta* (Ler, for the dcl1-9 mutant) and Columbia 0 (Col0, for the other mutants)

DNA constructs and generation of transgenic Arabidopsis plants

The *GFP-miR168** sensor construct was prepared as previously described (Parizotto et al. 2004). Essentially, the miR168 target sequence was introduced downstream of the *GFP* coding region by PCR. Amplified fusions were cloned into pGEM-T Easy and then inserted into pBIN61 binary vector under the control of the *35SCaMV* promoter. For preparation of the miR168 promoter-*GFP* construct, a 2kb fragment upstream of the miR168a precursor was amplified from Arabidopsis genomic DNA and cloned into pMDC110 binary vector upstream of the *GFP* reporter gene. Primers used for PCR are listed in Table S3. For plant transformation, the DNA constructs were mobilized into *Agrobacterium tumefaciens* strain GV3013 and introduced into Arabidopsis *sde1* mutants (C24 background) (Dalmay et al. 2000) by the floral dip method. Green fluorescence was recorded in whole transgenic plants with an Olympus SZX16 stereomicroscope, using 460-nm excitation and 510-nm emission filters coupled to a Digital color camera DP71 Olympus.

miRNA microarray analysis

For microarray analysis, three sets of RNA samples representing the three biological replicates for each time point (5min, 30 min, 60min and 120 min) and condition (control and elicitor-treated) were prepared. Equal amounts of total RNA from each biological replicate were pooled and used for isolation of the small RNA fraction using the miRVana™ miRNA Isolation kit (Ambion®).

The array contained 2,382 probes encompassing: 166 Arabidopsis miRNAs currently annotated in miRBase (http://microrna.sanger.ac.uk), 553 predicted miRNAs, and 1096 small RNA sequences generated by MPSS (Meyers et al. 2004a; Meyers et al. 2004b) (Table S1). In addition, a set of control probes complementary to known *C. elegans* and *D. melanogaster* miRNAs (134 and 78 entries, respectively) were included in the array.

Probe labeling and array hybridization were performed using the NCode™ miRNA Labeling System (Invitrogen, Cat#MIRLS-20), according to the manufacturer´s instructions. Hybridized arrays were scanned with a GenePix 4200A scanner (Axon Instruments, Forster City, California, United States) as previously described (Wellmer et al. 2004; Wellmer et al. 2006). Raw signal intensities for each probe set on each hybridized chip, contained in the .gpr files, were exported from the Gene Pix v.5.1 analysis software (Axon Instruments), and imported into Resolver Gene Expression Data Analysis System v.4.0 (www.rosettabio.com) for normalization and error correction. Averages of three biological replicates of normalized values were taken to calculate the expression ratios between elicitors and controls prior to $\log_{10}$ transformation. P-values for differential expression calculated by Rosetta Resolver Biosoftware were further adjusted for multi-hypothesis testing using the Benjamini & Hochberg procedure, as implemented in the Bioconductor *multtest* package in R (http://www.bioconductor.org/packages/bioc/stable/src/contrib/html/multtest.html) as described (Varkonyi-Gasic et al. 2007). Probes, for which the adjusted p-value was ≤ 0.05 were considered differentially expressed in the experiment. The microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE59978 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59978).

Gene expression analysis by quantitative reverse transcriptase-PCR (qRT-PCR)

Reverse transcription reactions were performed using total RNA from Arabidopsis seedlings. The same plant material used for small RNA extraction was also used for total RNA extraction. First-strand cDNA was synthesized from DNase-treated total RNA (3 µg) with SuperScript III reverse transcriptase (Invitrogen GmbH) and

oligo-dT_{18} (Qiagen, Hilden, Germany). The absence of contaminating genomic DNA after DNase1 treatment was confirmed by qRT-PCR analysis using primer pairs designed to amplify a 198 bp intron sequence of the *FLC1* gene. Primer sequences are indicated in Table S3. qRT-PCRs were performed in optical 96-well plates in a Light Cycler 480 (Roche) using SYBR® Green. Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The *Ubiquitin-10* (At5g65080) gene was used as the internal control for normalization. The average cycle threshold (Ct) values from triplicate PCRs were normalized to the average Ct values for the *Ubiquitin-10* gene from the same RNA preparations yielding the ΔCt value. Two independent biological replicates were analyzed. Controls of the qRT-PCR reactions without adding the reverse transcriptase enzyme were systematically included in our experiments.

Small RNA gel blot analysis

Total RNA was extracted using the TRIzol® reagent (Invitrogen™) according to the manufacturer's instructions. For small RNA Northern blot analysis, total RNAs were fractionated in a 17.5% polyacrylamide gel containing 8 M urea. As probes we used γ^{32}P-ATP end-labeled oligonucleotides complementary to the small RNA sequence under study. Probes used for Northern blot analysis are indicated in Table S3. Blots were pre-hybridized and hybridized in Perfect-Hyb Plus buffer (Sigma). Hybridization signals were detected using a STORM Phosphorimager (GE Helthcare).

Sequence analysis of *MIR168* promoters

The DNA sequences upstream of the start of the miRNA precursor structure of miR168a and miR168b were extracted from NCBI (http://www.ncbi.nlm.nih.gov). In each case, the 2kb upstream sequence from the start of the miRNA precursor was scanned for the presence of *cis*-elements using the web-based analysis tool PLACE, Plant Cis-acting Regulatory DNA Elements (http://www.dna.affrc.go.jp/PLACE). The overrepresentation of known promoter *cis*-elements and motifs was assessed using the POBO application (http://ekhidna.biocenter.helsinki.fi/poxo/pobo/pobo) (Kankainen

and Holm 2004). Statistical significance was calculated using the linked Graphpad application for a two-tailed comparison ($P \leq 0.0001$).

Supporting data

The microarray data obtained in this study have been deposited in NCBI's Gene Expression Omnibus (GEO) database under the accession number GSE59978 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59978).

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Author's contributions

PB carried out the experiments for the characterization of hs-siRNA415 and transgenic Arabidopsis plants, and contributed to data analysis. KK and CS performed expression studies on miR168/AGO1, microarray data analysis, and contributed to the production of transgenic plants. CS, ABM, AB and MGC participated in the preparation of RNA samples, plant transformation vectors and miR168 expression studies. JLR designed and conducted the microarray experiments. JJLM, JLR and BSS conceived the study. BSS wrote the paper, and JJLM and JLR made a critical revision of the manuscript. All authors read and approved the final manuscript.

Supplemental Materials

Additional file 1. Figure S1. Frequency of occurrence of defense-related *cis*-elements in the *MIR168a* promoter generated in POBO (http://ekhidna.biocenter.helsinki.fi/poxo/pobo/pobo)**. Figure S2.** Accumulation of siRNA415 in control (c) and elicitor-treated (e) Arabidopsis plants.

Additional file 2. Table S1. Arabidopsis small RNAs represented in the microarray. **Table S2**. *cis*-elements identified in the *MIR168a* and *MIR168b* promoters. **Table S3**. Sequences of oligonucleotides used in this study.

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Chapter II

MicroRNA-mediated regulation of gene expression in the response of rice plants to fungal elicitors

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that have important regulatory functions in plant growth, development, and response to abiotic stress. Increasing evidence also supports that plant miRNAs contribute to immune responses to pathogens. Here, we used deep sequencing of small RNA libraries for global identification of rice miRNAs that are regulated by fungal elicitors. We also describe 9 previously uncharacterized miRNAs in rice. Combined small RNA and degradome analyses revealed regulatory networks enriched in elicitor-regulated miRNAs supported by the identification of their corresponding target genes. Specifically, we identified an important number of miRNA/target gene pairs involved in small RNA pathways, including miRNA, heterochromatic and trans-acting siRNA pathways. We present evidence for miRNA/target gene pairs implicated in hormone signaling and cross-talk among hormone pathways having great potential in regulating rice immunity. Furthermore, we describe miRNA-mediated regulation of Conserved-Peptide upstream Open Reading Frame (CPuORF)-containing genes in rice, which suggests the existence of a novel regulatory network that integrates miRNA and CPuORF functions in plants. The knowledge gained in this study will help in understanding the underlying regulatory mechanisms of miRNAs in rice immunity and develop appropriate strategies for rice protection.

Introduction

Small RNAs (sRNAs) are short non-coding RNAs that guide gene silencing in most eukaryotes (Baulcombe 2004; Vaucheret 2006). Plants have two main classes of sRNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs), (Carrington and Ambros 2003; Voinnet 2009; Axtell 2013) with a major difference being their mode of biogenesis and/or function. MiRNAs are produced from single-stranded RNA precursors with unique stem-loop structures processed in two steps by a RNase III DICER-like (typically DCL1) to result in an miRNA-miRNA* duplex. After transfer to the cytoplasm, miRNA duplexes are loaded into the RNA-induced silencing complex (RISC), where ARGONAUTE 1 (AGO1) is the core component. However, siRNAs result from processing long perfect double-stranded RNA (dsRNA) precursors that are synthesized by the activity of a RNA-dependent RNA polymerase (RDR). Plant siRNAs can be further divided into heterochromatic siRNAs (hc-siRNAs), natural antisense siRNAs (nat-siRNAs), trans-acting siRNAs (ta-siRNAs), long small interfering RNAs (lsiRNAs) and phased secondary siRNAs (phasiRNAs) (Axtell 2013). The production and function of each class of sRNAs have consistent requirements for specific members of the DCL, RDR and AGO gene families. DCL1 and AGO1 are mainly involved in the miRNA pathway (Vaucheret et al. 2004).

MiRNAs regulate gene expression by triggering sequence-specific cleavage or translation repression of the target transcripts (Llave et al. 2002; Brodersen et al. 2008). In plants, miRNAs have a pivotal role in the regulation of gene expression during development (Palatnik et al. 2003; Chen 2012) and adaptation to a variety of abiotic stresses, such as drought, cold, salinity, and nutrient deficiency (Chiou et al. 2006; Sunkar et al. 2007; Jeong and Green 2013). Evidence is also accumulating for a role of miRNAs in the plant response to pathogen infection (Ruiz-Ferrer and Voinnet 2009; Katiyar-Agarwal and Jin 2010; Shivaprasad et al. 2012; Campo et al. 2013; Li et al. 2013; Staiger et al. 2013). The first indication of a miRNA playing a role in plant immunity came with the finding that treating Arabidopsis plants with the flagellin-derived elicitor peptide flg22 induced accumulation of miR393, which negatively regulates transcripts for the F-box auxin receptors (i.e., transport inhibitor response, TIR). MiR393-mediated repression of auxin signalling results in bacterial resistance (Navarro et al. 2006). However, our understanding of the functional roles of

miRNAs in biotic stress responses is far less than that in plant development and abiotic stress responses.

Most of the miRNAs reported in early articles are highly conserved throughout the plant kingdom and target transcription factors (Jones-Rhoades et al. 2006). Plants also express species-specific miRNAs that function in distinct biological processes and/or response to environmental stress (Fahlgren et al. 2007; Cuperus et al. 2011). Studies on pathogen-associated miRNAs have been conducted mainly in the interaction of Arabidopsis plants with the bacterial pathogen *Pseudomonas syringae* or treatment with the flg22 elicitor from *P. syringae*, but less is known about miRNAs mediating defence against fungal pathogens.

Plants possess a potent immune system for defence against most pathogens. Pathogenic organisms are recognized by conserved pathogen-associated molecular patterns (PAMPs, also known as elicitors) through pattern-recognition receptors (PRRs) located on the cell surface. Perception of PAMPs triggers basal defence, also known as PAMP-triggered immunity (PTI), which encompasses the immune responses against most pathogens (Jones and Dangl 2006). PTI components include deposition of callose, production of reactive oxygen species (ROS), protein phosphorylation processes, and induction of pathogenesis-related (PR) gene expression, among others. Moreover, the essential role of the phytohormones salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) in resistance to pathogens is well established in plants (Pieterse et al. 2012; Denancé et al. 2013). Successful pathogens have evolved mechanisms to counteract the basal defence, by delivering effector proteins into the plant cells that interfere with PTI (Chisholm et al. 2006). In turn, many plants have evolved another layer of immunity, the so-called effector-triggered immunity (ETI) consisting of proteins encoded by resistance (*R*) genes that specifically identify the corresponding effector proteins produced by the pathogen. Most studies of plant immunity have focused on the transcriptional regulation of protein-coding genes, and much less is known about miRNA-mediated regulatory processes. We have some examples of miRNAs that guide cleavage of disease resistance genes in *Solanaceae* and *Leguminosae* species (Jagadeeswaran et al. 2009; Zhai et al. 2011; Shivaprasad et al. 2012). More recently, an apple miRNA, Md-miRLn11, was reported to target a nucleotide-binding site leucine-rich repeat (NBS–LRR) protein that mediates resistance against apple leaf spot disease (Ma et al. 2014). The current scenario is that miRNAs are implicated in both PTI and ETI responses.

Rice (*O. sativa* L) is one of the most important cereal crops in the world and the main staple food crop for more than 50% of the world's population. The activity of certain miRNAs significantly affects traits of agronomic importance in rice, such as tiller growth, early flowering, panicle and grain production (Wang et al. 2012; Zhang et al. 2013). However, rice yields are severely compromised by the fungal pathogen *Magnaporthe oryzae,* the causal agent of the rice blast disease (Wilson and Talbot 2009). Indeed, rice blast is considered the most important fungus-caused disease in plants in terms of scientific and economic relevance (Dean et al. 2012). Both PTI and ETI are involved in immunity against the rice blast fungus (Liu et al. 2013). Although the rice/*M. oryzae* pathosystem has been extensively analyzed at the molecular and cytological levels, miRNA-mediated defence responses have not been fully described in this pathosystem. The function of distinct rice miRNAs in blast disease resistance has only recently been demonstrated, for miR160a, miR398b and miR7695 (Li et al. 2010a; Campo et al. 2013). Target genes for rice miRNAs have been mainly predicted by computational approaches, and only a small fraction of targets has been experimentally validated.

Owing to the scientific and economic importance of the rice/*M. oryzae* pathosystem, this study focused on rice miRNAs regulated by the rice blast fungus. We prepared sRNA and degradome libraries from rice tissues (leaves, roots) treated, or not treated with *M. oryzae* elicitors. Use of high-throughput Illumina sequencing revealed a comprehensive picture of the miRNA transcriptome in each tissue and the response of rice miRNAs to fungal elicitors. Furthermore, 9 novel miRNAs from rice were identified. A degradome approach was used to identify transcriptome-wide miRNA targets in rice. An interesting observation was the identification of miRNA-guided cleavage of Conserved Peptide upstream Open Reading Frames (CPuORF)-containing transcripts. We provide evidence for miRNA-mediated regulatory networks controlling sRNA biogenesis and functioning, and hormone signalling cross-talk in the rice response to fungal elicitors. The data will serve as foundation for studies addressing fundamental molecular mechanisms that govern rice immunity.

Results

Genome-wide expression profiling of rice miRNAs

To obtain a genome-wide survey of miRNAs in rice and their responsiveness to fungal elicitors, we constructed sRNA libraries from rice leaves and roots treated or not with elicitors obtained from the rice blast fungus *M. oryzae*. Elicitors are widely used to trigger typical defense responses in numerous plant species, including rice (Schaffrath et al. 1995). Elicitor-treated and mock-treated tissues were harvested at two times of elicitor treatment (30 min and 2 h). Illumina Solexa sequencing of sRNA libraries generated 118,810,219 reads (approx. 62.5 and 56.3 million reads from leaf and root libraries, respectively) (Supplemental Figure 1A). After trimming the adapter sequences and short sequences, a total of 47,866,683 and 41,541,308 mappable sRNA sequences were obtained from leaves and roots, respectively. SRNAs then underwent a BLAST search against the known non-coding RNA families (ribosomal RNAs, transfer RNAs, small nuclear RNAs and small nucleolar RNAs) deposited in the Rfam Genbank databases, and distinct sRNAs belonging to these categories were removed. For details about the bioinformatic analysis of sequencing data, see Supplementary Methods. A total of 7,660,460 and 3,022,421 unique sequences were generated from leaf and root libraries, respectively (Supplemental Figure 1A). Consistent with the typical sRNA distribution in plants, most of the sRNAs were 18-27 nt in length in the two tissues. The highest abundance was found for 24-nt sequences in terms of both total abundance and unique sequences (Supplemental Figure 1B, C).

SRNA sequences perfectly mapping the rice genome were searched against the miRBase database (version 21, http://www.mirbase.org (Kozomara and Griffiths-Jones 2014)). Currently, miRBase has 713 registered rice miRNAs representing 334 families. Following the current annotation, the mature miRNAs identified in our sequencing dataset originating from opposite arms of the same pre-miRNA are denoted with a -5p or -3p suffix (miRNA or miRNA* species). A total of 705 miRNAs and miRNAs* representing 332 known miRNA families were identified in leaf libraries (Supplemental Table 1). In roots, 696 miRNAs corresponding to 326 miRNA families were detected (Supplemental Table 1). Thus, the sequencing depth obtained in this study was sufficient for a comprehensive differential expression analysis of rice miRNAs and presumably also for the identification of novel miRNAs from rice.

By analysing the Illumina sequencing dataset, we obtained the expression profile of rice miRNAs in each tissue (control libraries). Members of the miR166, miR168, and miR396 families were the most abundant in rice leaves (Figure 1A, left panel). In roots, miR166 family members and miR2863b showed the highest expression (Figure 1A, right panel). However, miR2863 accumulated at a relatively low level in leaves (Supplemental Table 1). Differences in the expression of miRNAs and/or among members of a particular miRNA family in one or another tissue might indicate a functional divergence in these tissues.

To validate the expression pattern of miRNAs obtained by deep sequencing, we randomly selected 8 miRNAs with differential accumulation in leaf or root rice tissues.

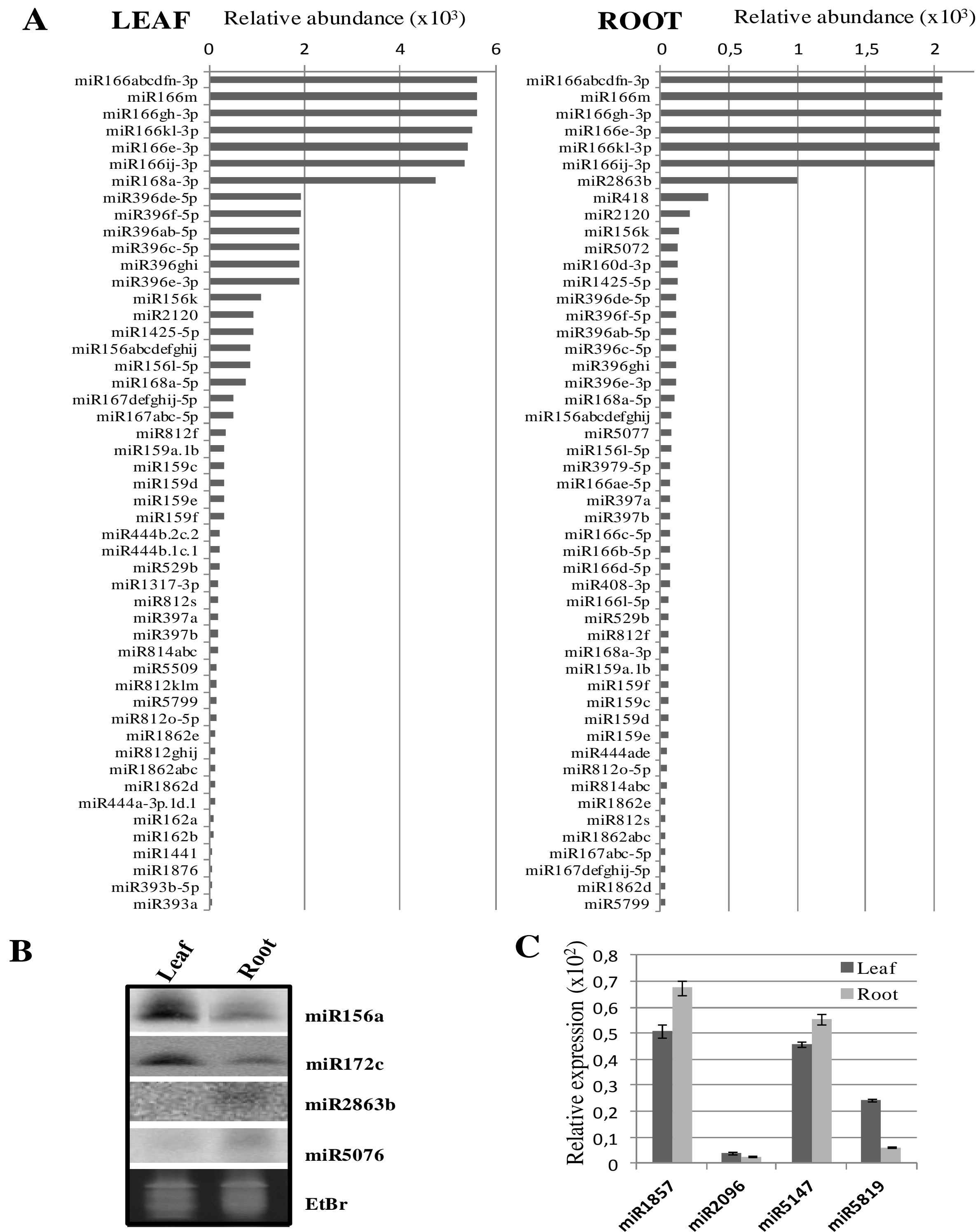


Figure 7: Expression profiling of known miRNAs from rice. (A) Expression of known miRNAs in leaves and roots of rice plants. Reads retrieved from the Solexa/Illumina sequencing dataset for each family member in control libraries were normalized to the total count of reads obtained in the corresponding library. Only the most abundantly expressed miRNAs are presented. Asterisks denote miRNAs examined in B and C. Details on miRNA expression in each tissue are in Supplemental Table 1. (B, C) Expression of miRNAs identified in small RNA libraries from rice tissues by Northern blot analysis (B) or stem-loop RT-qPCR (C). Lower panel in (B) shows ethidium bromide staining of RNA samples. Oligonucleotides used as probes in (B) are indicated on the right side.

Northern blot analysis confirmed the expression of miRNAs showing high and moderate abundance in our sequencing dataset (e.g., miR156a, miR172c, miR2863b, miR5076), whereas the expression of low-abundant miRNAs (e.g., miR1857, miR2096, miR5147, miR5819) was validated by stem-loop RT-qPCR followed by nucleotide sequencing (Figure 1C; details on miRNA abundance are in Supplemental Table 1). According to the miRBase registry (release 21), the low-abundant miRNAs (miR1857, miR2096, miR5147, miR5819) are identified only in rice.

A detailed analysis of Illumina sequencing data of sRNA libraries also revealed the presence of miRNA sequences representing new members of known miRNA families. They were named as miR1861p, miR2120b, miR5801c, and miR6245b (Figure 2; the nucleotide sequence of these precursors is in Supplemental Figure 2).

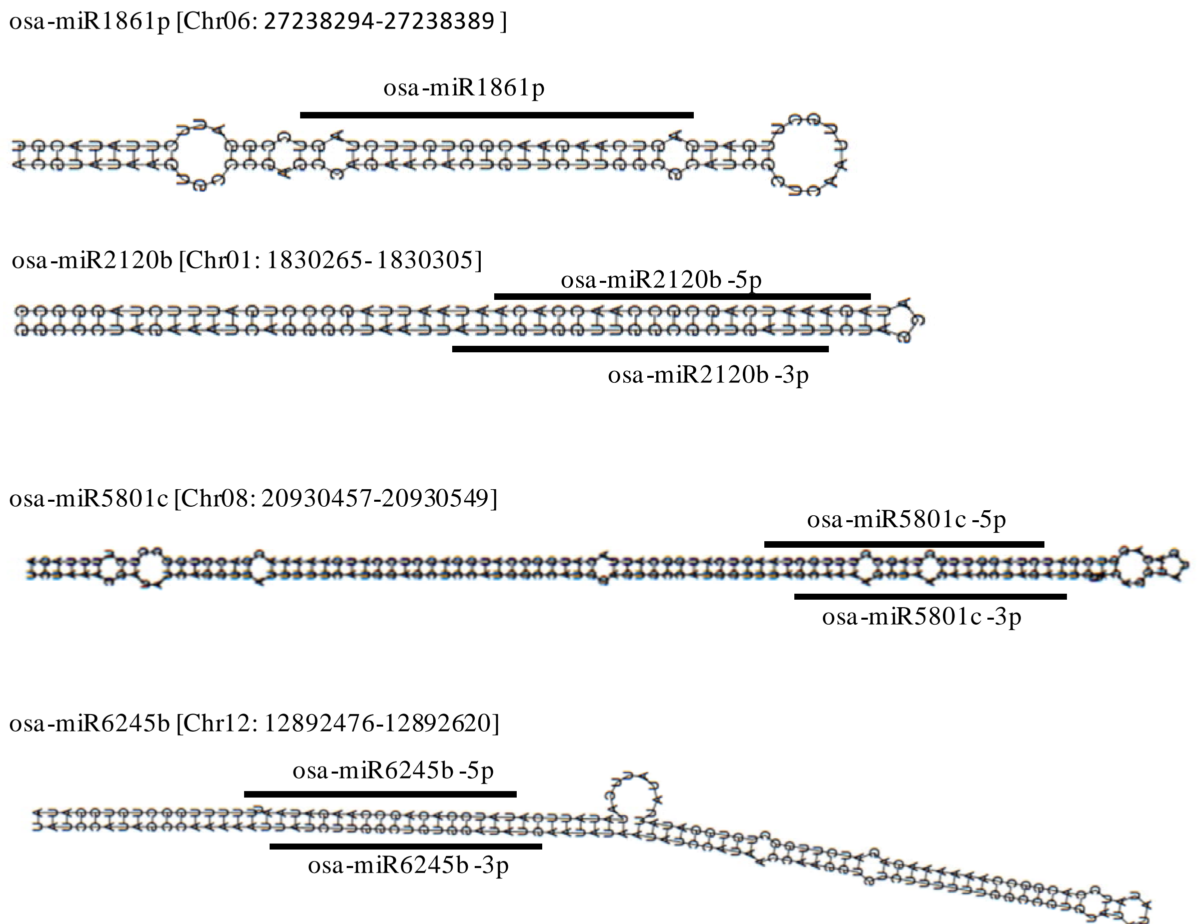


Figure 8: Precursor structures of novel members of known miRNA families. Small RNA sequences recovered from the Solexa/Illumina sequencing data mapping into these structures are represented by black bars. The nucleotide sequences of these precursor structures are in Supplemental Figure 2.

Identification of novel miRNAs from rice

The identification of previously uncharacterized miRNAs from rice was a major objective of this study. Towards this end, we used the miRDeep-P software with default parameters (Yang and Li 2011). Briefly, we computationally predicted miRNA stem loop precursor structures and searched for sRNAs mapping opposite to each other at both strands of the hairpin (e.g., miRNA/miRNA* sequences) having the characteristic 2-nt 3'-overhangs, a signature of DICER cleavage (Meyers et al. 2008; Kozomara and Griffiths-Jones 2014). Mismatches between sRNA reads and the rice genome were not allowed. In this way, we identified 9 loci that fulfilled the fold-back structure criterion for miRNA precursors (Figure 3A; precursor nucleotide sequences are in Supplemental Figure 3; expression data are in Supplemental Table 2). The names assigned in the miRBase registry for the novel miRNAs identified in this work were osa-miR11336, osa-miR11337, osa-miR11338, osa-miR11339, osa-miR11340, osa-miR11341, osa-miR11342, osa-miR11343 and osa-miR11344.

SRNA Northern blot analysis was carried out to validate the newly identified miRNAs from rice (Figure 3B). Both miRNA-5p and miRNA-3p species were detected for 8 of the 9 miRNAs identified, which further supports that they represent previously uncharacterized miRNAs from rice. These miRNAs accumulated at relatively low levels in rice tissues as judged by both the low number of reads found in the Illumina sequencing data (Supplemental Table 2), and the large amount of RNA needed for their detection by Northern blot analysis. Five of the novel miRNAs located in intergenic regions, whereas 4 mapped to the intronic region of a gene (Figure 3A). None of these novel miRNAs have obvious orthologs in any other plant species for which genomic sequences are available (NCBI database) suggesting that they might represent novel rice-specific miRNAs.

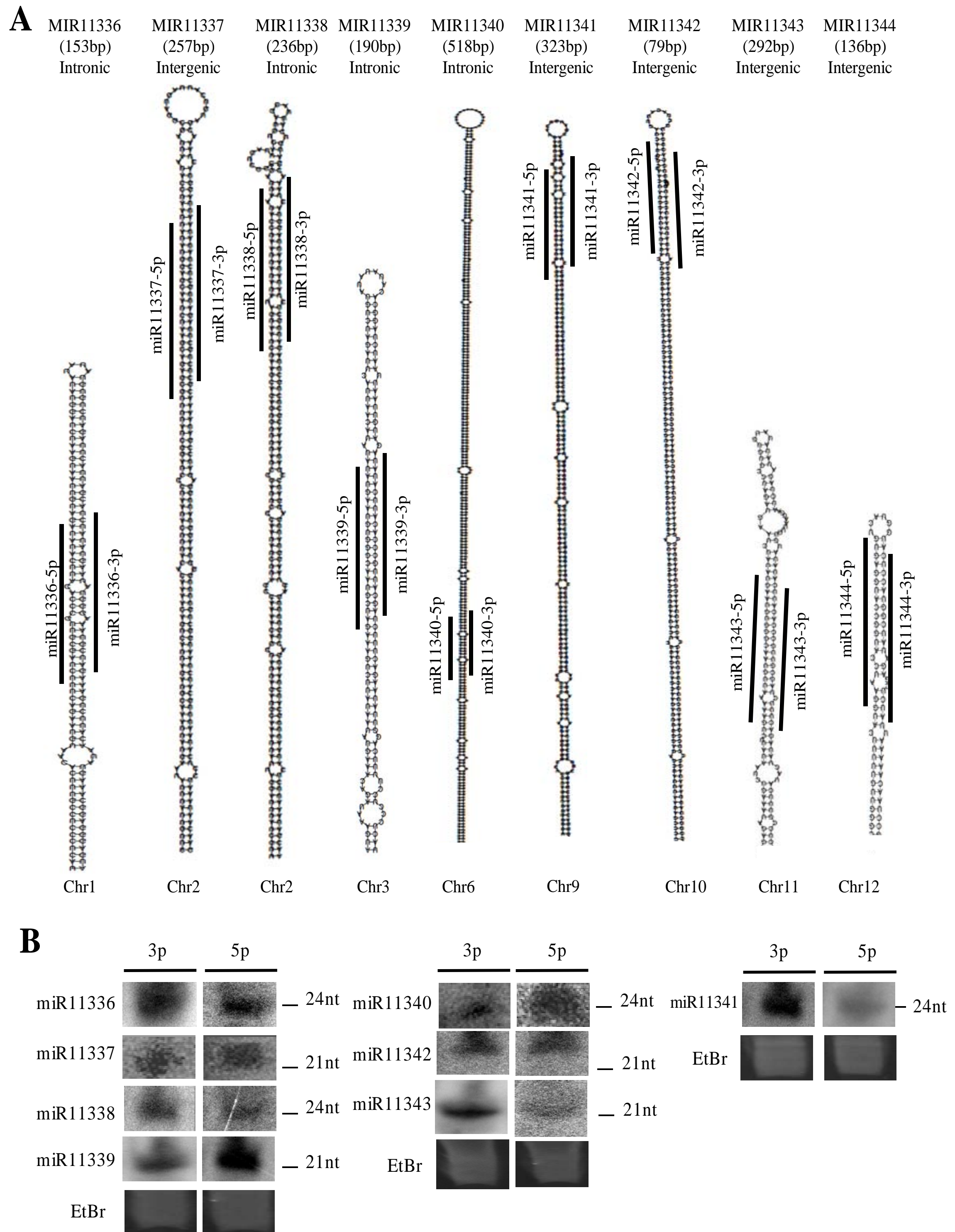


Figure 9 Precursor structures and detection of novel miRNAs from rice. (**A**) Precursor structures of novel miRNAs. Small RNA sequences mapping into these structures are represented by black bars. Additional information on the nucleotide sequence and chromosomal location is in Supplemental Figure 3. (**B**) Northern blot analysis of novel miRNAs**.** Total RNA samples (200 µg) were analysed. The same blots were successively stripped and re-probed with ^{32}P-end-labelled oligonucleotides. Except for miR11341, which was detected in roots, all novel miRNAs accumulated in rice leaves. Lower panels show ethidium bromide staining of RNA samples.

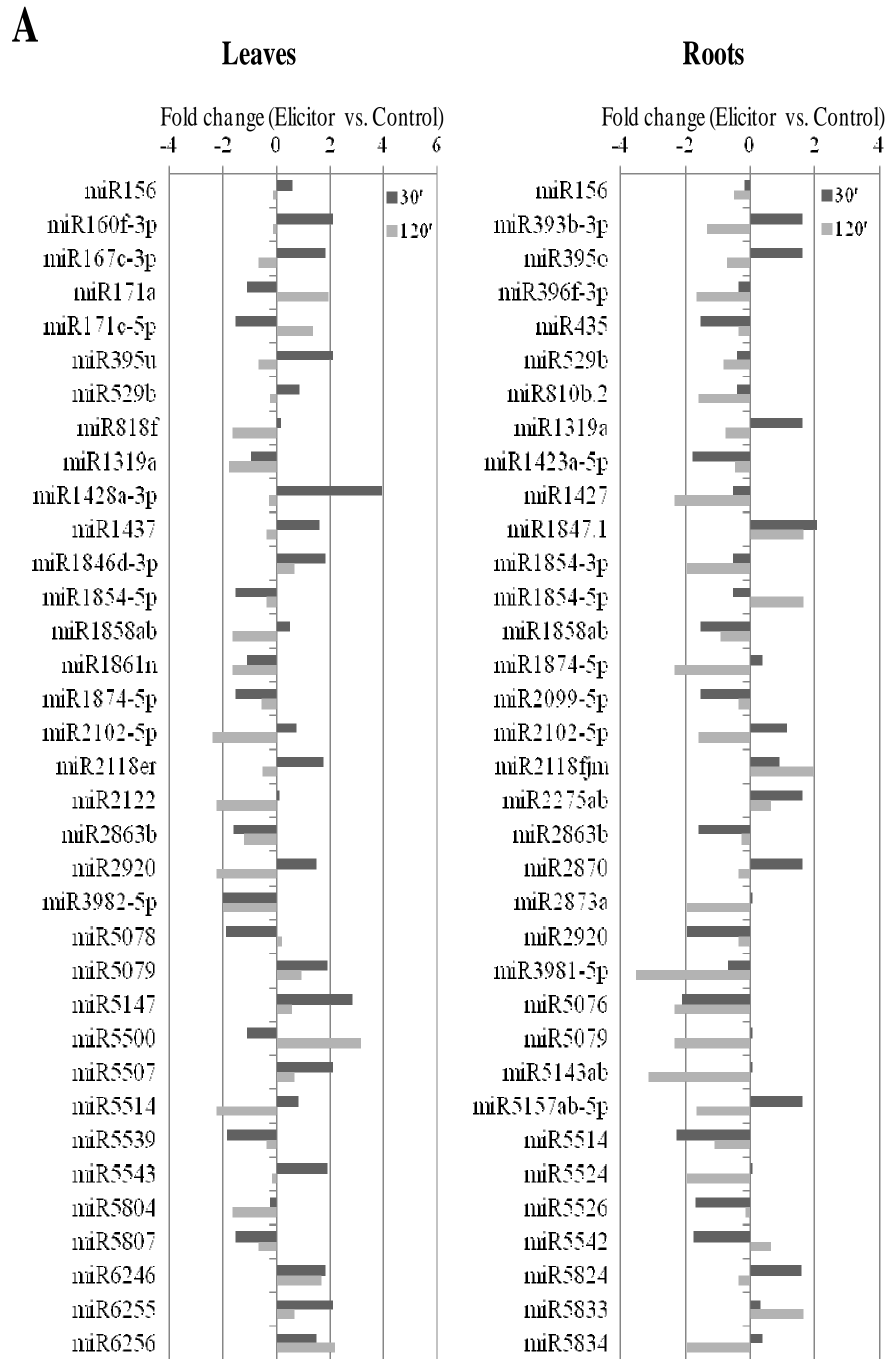
Elicitor-responsiveness of rice miRNAs

The sequencing frequencies for miRNAs in our sRNA libraries, as calculated from normalized libraries, were used to investigate the elicitor-responsiveness of rice miRNAs. Figure 4A shows representative examples of elicitor-induced alterations in accumulation of known miRNAs (detailed information on the abundance for all known miRNAs identified in each tissue and condition is in Supplemental Table 1). Consistent with the role of miRNAs as modulators of gene expression, a dynamic response occurs on the accumulation of rice miRNAs in response to fungal elicitors. In some cases, the elicitor-responsiveness of a particular miRNA showed the same trend of expression in the two tissues (e.g., miR2863b, down-regulated), whereas in other cases, a miRNA showed a different response to elicitors depending on the tissue (e.g., miR6255, up-regulated in leaves but down-regulated in roots) (Figure 4A). Also, a different response could be observed for a particular miRNA at one or another time of elicitor treatment.

The elicitor-responsiveness of known miRNAs accumulating at high or moderate level in rice tissues (as judged by the number of reads in the sRNA sequencing dataset) was further validated by Northern blot analysis (Figure 4B). These miRNAs included miR156a, miR529b and miR5078. A similar trend in the response to elicitors was observed when comparing results obtained by Northern blot analysis and sequencing data. Like known miRNAs, the newly identified miRNAs also showed a dynamic response to treatment with fungal elicitors (Supplemental Figure 4; Supplemental Table 2).

Collectively, analysis of sRNA deep sequencing data revealed alterations in the accumulation of an important number of rice miRNAs in response to treatment with *M. oryzae* elicitors.

Figure 10: Elicitor-responsiveness of known miRNAs from rice. (**A**) Expression analysis of known miRNAs in leaves and roots at 30 min or 2 h of elicitor treatment (light and dark bars, respectively) as determined by the logarithm of fold change (elicitor *vs*. control). Representative examples are shown (see Supplemental Table 1 for detailed information on the expression of the complete list of rice miRNAs). Asterisks denote miRNAs examined in **B**. (**B**) Northern blot analysis of miR156a, miR529b and miR5078 in control and elicitor-treated rice leaves. Total RNA samples (70 µg) were analysed. Oligonucleotides used as probes are indicated on the right. c, control; e, elicitor.


A
Leaves
Roots
Fold change (Elicitor vs. Control)
-4 -2 0 2 4 6
-4 -2 0 2 4
30'
120'
miR156
miR160f-3p
miR167c-3p
miR171a
miR171c-5p
miR395u
miR529b
miR818f
miR1319a
miR1428a-3p
miR1437
miR1846d-3p
miR1854-5p
miR1858ab
miR1861n
miR1874-5p
miR2102-5p
miR2118er
miR2122
miR2863b
miR2920
miR3982-5p
miR5078
miR5079
miR5147
miR5500
miR5507
miR5514
miR5539
miR5543
miR5804
miR5807
miR6246
miR6255
miR6256
miR156
miR393b-3p
miR395c
miR396f-3p
miR435
miR529b
miR810b.2
miR1319a
miR1423a-5p
miR1427
miR1847.1
miR1854-3p
miR1854-5p
miR1858ab
miR1874-5p
miR2099-5p
miR2102-5p
miR2118fjm
miR2275ab
miR2863b
miR2870
miR2873a
miR2920
miR3981-5p
miR5076
miR5079
miR5143ab
miR5157ab-5p
miR5514
miR5524
miR5526
miR5542
miR5824
miR5833
miR5834

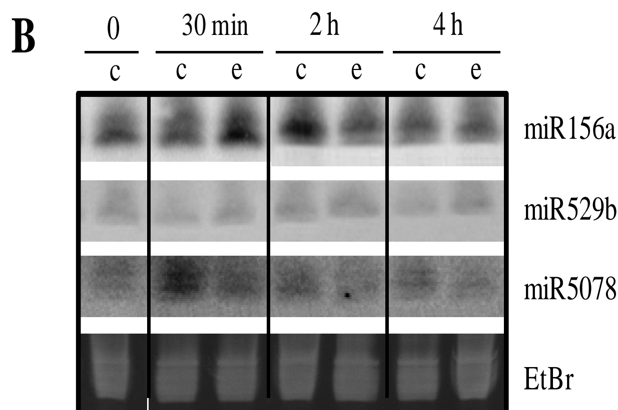

B
0
30 min
2 h
4 h
c
c e
c e
c e
miR156a
miR529b
miR5078
EtBr

Table 3 Target genes for known miRNAs from rice identified by degradome analysis.

miRNA [(1)]	Target gene for -5p	miRNA [(1)]	Target gene for -3p
miR156	SBP domain containing protein	**miR156**	
	(OsSPL2 - OsSPL3 - OsSPL4 - OsSPL11 - OsSPL12 -	**(b)**	retrotransposon centromere-specific
	OsSPL13 - OsSPL14 - OsSPL17 - OsSPL18 - OsSPL19)		
	DUF260 domain containing protein		
	O-methyltransferase		
miR159	MYB family TF		
(a, b, c, d, e, f)	MAP kinase 8		
miR160	Auxin Response Factor (ARF8 - ARF10 - ARF18 - ARF22)	**miR160**	Receptor like kinase (CrRLK1L-1)
(bcef)		**(e)**	
miR162	Dicer-Like 1a (DCL1a)		
miR164	Transport protein Sec61, subunit alpha		
(d, e)	Resistance to *P.syringae* 1A (RGH1A)		
miR166	Chaperonin	**miR166**	START domain-containing protein (HOX9 - HOX10 -
(b, c, d)	Acetyltransferase, GNAT family		HOX32 - HOX33)
miR167	Auxin Response Factor 6 (ARF6)	**miR167**	Eukaryotic peptide chain release factor subunit 1-1
(a, b, c, e, f, g)	NBS-LRR disease resistance protein	**(d)**	
miR168	ARGONAUTE protein (AGO1a - AGO1b - AGO1c)		
miR171 (a, b, c, d, i)	AP2 domain containing TF (ERF#043 - ERF#073 - ERF#090)		
miR172	Auxin Response Factor 9 (ARF9)	**miR172**	Spotted leaf 11
(b)	ERF#033 - AP2 domain containing TF	**(a, d)**	Haloacid dehalogenase-like hydrolase family protein
miR393	Transport inhibitor response 1 (TIR1 - OsFBL21)	**miR393**	Hydroxyacid oxidase 1
(a, b)	F-box and LRR containing protein - (OsAFB2 - OsFBL16)	**(b)**	
	Supressor of Gene Silencing 3 (SGS3) (leafbladeless1)		
miR396	Clathrin assembly protein	**miR396**	Outer mitochondrial membrane porin
(a, b, c)	Growth regulating factor (OsGRF3 - OsGRF4 - OsGRF5 - OsGRF10)	**(a, b)**	Ubiquitin carboxyl-terminal hydrolase
miR398 (a, b)	copper/zinc superoxide dismutase (SOD1 - SOD2)		
miR408	Expressed protein	**miR408**	Plastocyanin-like domain containing protein
miR426	Chitinase 3 (CHIT3)		
miR437	Ethylene-responsive TF (TINY) (ERF)		
miR444	Protein disulfide isomerase PDIL2-1	**miR444**	DnaK family protein (Mitochondrial HSP70 2)
(a, d, e)	Glutamate receptor 2.8 precursor	**(a, d)**	
	Remorin		
miR528	L-ascorbate oxidase precursor	**miR528**	Nitrogen regulatory protein P-II
miR530	Cyclin-T1-2	**miR530**	EREBP#056 - AP2 domain containing TF
	Loricrin		
miR531a	Glutaredoxin subgroup III (OsGrx_S2)		
miR818	Pentatricopeptide		
(c, d)	RNA-dependent RNA polymerase 2 (RDR2)		
miR1319a	Lipid Transfer Protein family protein (LTPL6)		
miR1320	Anthocyanidin 3-O-glucosyltransferase	**miR1320**	Cyclophilin
miR1425	Rf1, mitochondrial precursor	**miR1425**	glyoxalase family protein
miR1426	ERF#090 - AP2 domain containing TF		
miR1428	HSF-type DNA-binding domain containing protein	**miR1428**	DUF584 domain containing protein
miR1439	D-mannose binding lectin family protein		
miR1439	NBS-LRR class disease resistance protein (NB-ARC)		
miR1439	MATE domain containing protein		
miR1439	Transferase family protein		
miR1846	Zinc finger, C3HC4 type domain containing TF	**miR1846**	RelA-SpoT like protein RSH4 (CRSH2)
miR1847.2	Argonaute 4b (AGO4b)		
miR1850.2	NBS-LRR class disease resistance protein (NB-ARC)		
miR1850.2	RNA-dependent RNA polymerase 2 (RdR2)		
miR1861c	Glyoxalase family protein		
miR2098	WD-40 repeat family protein	**miR2098**	DnaJ domain containing protein
miR2101	CPuORF7 containing SAM decarboxylase	**miR2101**	Phospholipid-transporting ATPase 2
miR2102	EREBP#056 - AP2 domain containing TF	**miR2102**	Expressed protein

Table 1 (continued): Target genes for known miRNAs from rice identified by degradome analysis

miRNA [1]	Target gene for -5p	miRNA [1]	Target gene for -3p
miR2871	Acyl carrier protein	**miR2871**	Glycosyltransferase family 43 protein
			Zinc finger, C3HC4 type, domain containing TF
miR2921	Leucine-rich repeat family protein		
miR2925	Remorin		
miR5075	Auxin Response Factor (ARF20)		
	CPuORF4 containing bZIP27 TF		
miR5077	ZIM domain containing protein (TIFY10B)		
miR5488	F-box domain and LRR containing protein (OsFBL5)		
miR5489	Pollen Ole e I allergen and extensin family protein (POEI27)		
miR5500	Alpha-N-arabinofuranosidase		
miR5512	Salicylic acid carboxyl methyltransferase (SAMT)		
miR5518	Glycosyl hydrolases family 16		
miR5530	Cyclophilin FKBP-type		
miR5793	EREBP#124 - AP2 domain containing TF		
miR5794	Rapid ALkalinization Factor (RALFL21)		
miR5809	STRESS ENHANCED PROTEIN 2 (SEP2)		
	Indole-3-acetic acid-amido synthetase (OsGH3.4)		
miR5819	Lipid Transfer Protein (LTPL118)		
	CPuORF3 containing bZIP38 TF		
miR6249	Jacalin-like lectin domain containing protein		

Only selected target genes classified into categories 0, 1 or 2 are shown (the complete list of target genes is presented in Supplemental Table 4). In bold, miRNAs for which target genes were identified for both the miRNA-5p and miRNA-3p sequences. [1] Letters below indicate the specific members for each miRNA family identified in the small RNA sequencing data set.

Identification of target genes of elicitor-regulated rice miRNAs

Most plant miRNAs have extensive complementarity to their target genes and regulate their expression predominantly through mRNA cleavage. The slicing activity on their target typically occurs between the nucleotides 10 and 11 from the 5’ of the miRNA, and the resulting 3’ fragment of the target mRNA possesses a free 5’ monophosphate. These cleavage products can be recovered by RNA ligase-mediated ligation, whereas the full-length cDNA with a 5’ cap structure or other RNAs lacking the 5’ monophosphate group are not compatible for ligation. This property was exploited to validate miRNA-mediated cleavage of target transcripts (Llave et al. 2002). Later on, a degradome sequencing technology was developed for high-throughput miRNA target identification in plant species (Addo-quaye et al. 2009; German et al. 2009).

To identify target genes of elicitor-regulated miRNAs, we generated degradome libraries from both control and elicitor-treated rice leaves (30 min and 2 h of treatment; same plant material used for construction of the sRNA libraries). Illumina sequencing of degradome libraries yielded 128.3 million reads, of which 51,568 unique signatures could be mapped to the rice transcriptome (statistics of degradome sequencing are in Supplemental Table 3). The miRNA cleavage sites were identified by using the PAREsnip platform developed for discovery of miRNA/target interactions evidenced through degradome sequencing (Folkes et al. 2012). The workflow used to identify miRNA targets is in Figure 5A. To visualize the cleavage events within the target mRNAs, we plotted the abundance of each signature as a function of its position in the target transcript (target-plots, or t-plots; representative t-plots are in Supplemental Figure 5A). The identified targets were classified into five categories based on the relative abundance of signatures at the target site and along the transcript (categories 0, 1, 2, 3 and 4) (Folkes et al. 2012). This analysis identified 602 targets for 299 of the 332 miRBase-annotated rice miRNA families. Transcripts showing high abundance reads at potential cleavage sites, described as categories 0, 1 and 2, represent the strongest evidence for true cleavage products (Folkes et al. 2012). Accordingly, in this work we considered only targets in categories 0, 1 and 2. Representative miRNA targets confirmed by degradome analysis for known rice miRNAs are in Table 1 (the complete list of target genes in categories 0, 1 or 2 is in Supplemental Table 4).

Among the miRNA targets identified by degradome sequencing, signatures associated with conserved miRNA targets were the most abundantly represented. Most were classified as category 0, thus confirming the accuracy of our degradome analysis (Supplemental Figure 6).

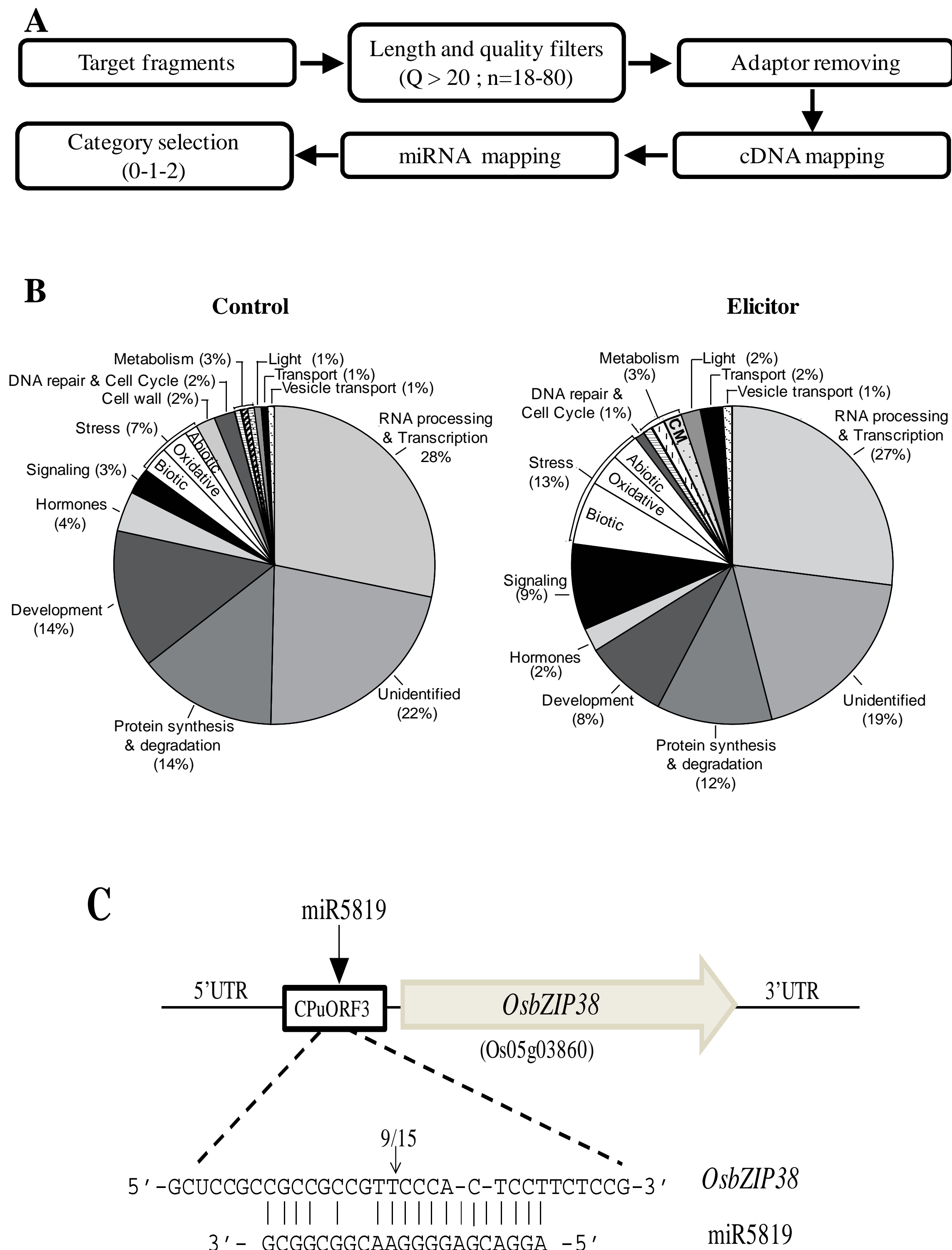


Figure 11: Confirmed target genes for rice miRNA using degradome sequencing. (A) Pipeline for target identification in degradome sequencing. (B) MapMan annotation of miRNA targets validated by degradome analysis in control and elicitor-treated leaves. Only the validated target genes identified by degradome sequencing in categories 0, 1 and 2 were considered. CM, carbohydrate metabolism.

Conserved miRNAs targeted several family members (e.g., miR156, miR164 and miR169), whereas non-conserved miRNAs tended to target few genes (e.g., miR2104, miR5075, miR5518 and miR5809). Cleavage events corresponding to non-conserved targets mediated by conserved miRNAs were also discovered. For instance, besides *SPL* genes, miR156b target genes encoding a DUF260 domain-containing protein and O-methyltransferase (Supplemental Table 4). MiR159abcdef targeted *MAP kinase 8* in addition to MYB transcription factor genes. Degradome analysis also revealed an NBS-LRR disease resistance gene as the target gene for miR167. Therefore, besides having conserved targets, deeply conserved miRNAs might also have specialized functions by regulating non-conserved targets. Similar to reports by other authors (Pantaleo et al. 2010; Li et al. 2010b), we found no clear association between the miRNA level and the cleavage frequency of target transcripts.

For a functional characterization of all miRNA targets identified, we performed a MapMan analysis (Thimm et al. 2004) of validated target genes for miRNAs identified in control or elicitor-treated rice tissues. In elicitor-treated tissues, genes targeted by miRNAs showed a strong enrichment in the subcategory of biotic stress (Figure 5B). Among the target genes with known functions in biotic stress responses were chitinase (targeted by miR426), lipid transfer protein (LTP, targeted by miR5819), disease resistance (targeted by miR164e, miR167, miR1439 and miR1850), and a D-mannose binding lectin protein (targeted by miR1439) (Table 1). Other target genes of note are those involved in detoxification systems, such as copper/zinc superoxide dismutase (SOD1 and SOD2, targeted by miR398), L-ascorbate oxidase (targeted by miR528), glutaredoxin (targeted by miR531a), and glyoxalase (targeted by miR1425 and miR1861c) (Table 1).

MapMan analysis revealed an increase in miRNA target genes classified as “Signaling” in elicitor-treated tissues compared to non-treated tissues (3% in control tissues; 9% in elicitor-treated tissues), with a decrease in those in the category of “Development” (14% in control tissues; 8% in elicitor-treated tissues). The functional category of carbohydrate metabolism was enriched in elicitor-treated but not control tissues. Likely, the target genes identified in degradome libraries are components of the elicitor-induced signal transduction pathways leading to activation of defense responses.

Of note, miR393 is well known to cleave the TIR/AFB2 clade of auxin receptors during PTI against *P. syringae* in Arabidopsis plants (Navarro et al. 2006). Consistent with this

finding, *TIR1* and *AFB2* (*OsFBL21* and *OsFBL16*, respectively) were found as targets of miR393 in our degradome analysis (Table 1). Interestingly, we found that, in addition to *TIR1* and *AFB2*, miR393 also cleaves *Suppressor of gene silencing 3* (*SGS3*) transcripts (Table 1). A soybean homolog of Arabidopsis SGS3 was previously reported as the target of a soybean miRNA (Soy_25) (Song et al. 2011).

Degradome analysis revealed targets for both the miRNA and miRNA* (i.e., miRNA-5p and miRNA-3p sequences) for 19 miRNAs supporting that their miRNA* sequences may be functional (Table 1). Some examples are miR160e, miR393b, miR530, miR1428e and miR2098. Moreover, target analysis revealed the regulation of distinct mRNA targets by two different miRNAs (Supplemental Table 5). These targets included transcripts encoding RDR2 (RNA-dependent RNA polymerase 2) (targeted by miR818cd and miR1850.2, AP2 domain-containing protein (targeted by miR171abcdi and miR1426), tyrosine protein kinase (targeted by miR818cd and miR1436), glyoxalase (targeted by miR1425 and miR1861c), and a transcriptional regulator (targeted by miR531 and miR5075). Co-regulation of target transcripts by two or more miRNAs was previously reported (Li et al. 2010b). However, whether the miRNAs targeting the same gene identified in this work act in concert to regulate target gene expression remains to be determined, as does whether cleavage of their corresponding target transcripts is biologically relevant or merely a neutral event.

Regulation of CPuORF-containing transcripts by rice miRNAs

Upstream open reading frame (uORF)-containing genes represent a specific class of selectively translated genes in which a short peptide (sPEPs) encoded by the uORF sequence modulates translation of the downstream major ORF. Typically, uORFs lie upstream of the main protein coding region (i.e., in the 5’ UT region of the mRNA). Although uORFs are a common feature in many eukaryotic mRNAs, including plants, those encoding conserved uORFs (CPuORFs) are relatively rare and occur in less than 1% of angiosperms (Hayden and Jorgensen 2007; Jorgensen and Dorantes-Acosta 2012) .

Of interest, three CPuORF-containing genes were identified among the target genes for rice miRNAs. These genes include CPuORF3-*OsbZIP38* (targeted by miR5819), CPuORF4-*OsbZIP27* (targeted by miR5075), and CPuORF7-SAM decarboxylase (targeted by miR2101) (Table 1; Supplemental Figure 7). Of them, miR5819 cleaves *OsbZIP38* transcripts within the

nucleotide sequence corresponding to the short peptide encoded by CPuORF3, which is located at the 5' UT region of *OsbZIP38* (Figure 5C). Consistent with results obtained by degradome analysis, 5'-RACE identified cleavage fragments at the expected site of *CPuORF3-bZIP38* transcripts, which further supports that these transcripts are cleaved by miR5819 (Figure 5C). The cleavage site for miR5075 and miR2101 located at the coding region of *OsbZIP27* or the 3'-UT region of *SAM decarboxylase*, respectively (Supplemental Figure 7). The three miRNAs targeting CPuORF-containing genes (miR5819, miR5075, miR2101) are rice-specific according to the current miRBase registry (release 21).

Pathways regulated by elicitor-responsive miRNAs in rice

Combined sRNA and degradome analysis allowed us to obtain a comprehensive list of miRNA/target interaction pairs in rice and revealed interesting regulatory networks mediated by elicitor-responsive miRNAs. We provide evidence for an important number of miRNAs (and their corresponding target genes) involved in sRNA biogenesis and functioning machinery, including the miRNA pathway (miR162/DCL1; miR168/AGO1), hc-siRNA pathway (miR818 and miR1850/RDR2; miR1847/AGO4), and ta-siRNA pathway (miR168/AGO1 and miR393/SGS3) (Figure 6A). As well, we identified miRNA/target gene pairs involved in several subnetworks associated with hormone signaling and crosstalk between defense-related hormones, namely ET, SA, JA and auxin signaling as well as polyamine biosynthesis (Figure 6B, C). For instance, we detected a miR1846-guided regulation of *ACC oxidase* (*ACO*; final step of ethylene biosynthesis), and several miRNAs that regulate the expression of ethylene-responsive genes (ERF33, ERF43, ERF73, ERF90, EREBP56, and EREBP124) (Figure 6B). Furthermore, we identified distinct miRNA/target gene pairs controlling the conversion of S-adenosyl-L-methionine (SAM) to the specific precursor molecules required for the production of Me-SA, Me-JA, or polyamines. These signaling pathways are connected with the ethylene signaling pathway through SAM. These findings will be discussed in more detail below.

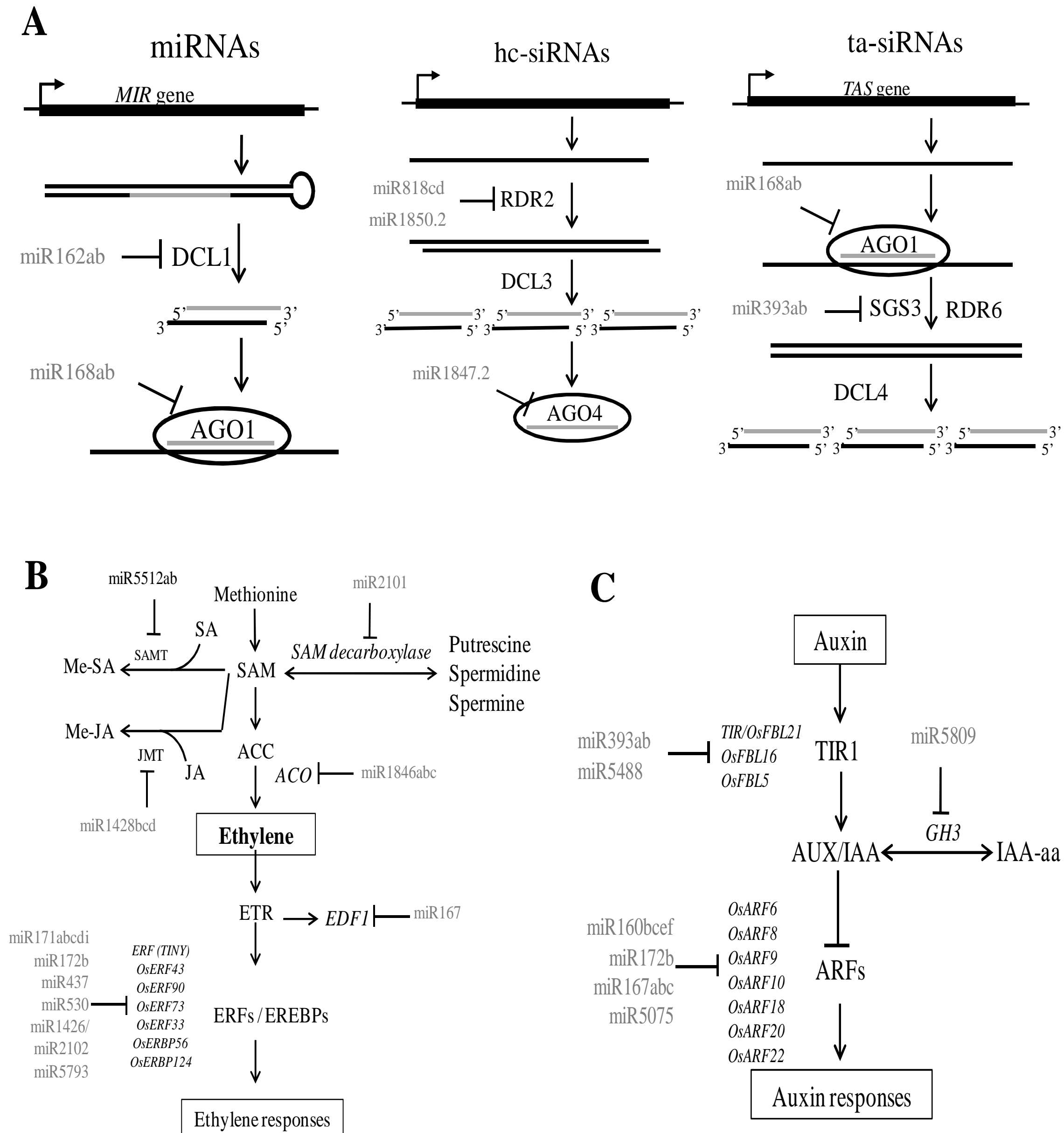


Figure 12: Overview of regulatory networks in which miRNA/target pairs function during the rice response to fungal elicitors. Small RNA and degradome sequencing data were used to establish regulations in the indicated pathways. All target genes were supported by degradome sequencing. miRNAs targeting these genes are boxed. Except for *EDF1*, all the indicated target genes are classified in categories 0, 1 or 2 in the degradome analysis. (Folkes et al. 2012) (**A**) Small RNA biogenesis and functioning. AGO, ARGONAUTE; DCL, DICER-like; RDR, RNA-dependent RNA polymerase; SGS3, Suppressor of gene silencing. (**B**) Ethylene signaling pathway and crosstalk with salcylic-acid and jasmonic-acid signaling pathways, and polyamine biosynthesis. EDF1, ethylene response DNA binding factor 1; EREBP, ethylene-responsive element binding protein; ERF, ethylene responsive factor; ETR, ethylene receptor; JMT, jasmonic acid carboxyl methyltransferase; Me-JA, methyl-jasmonic acid; Me-SA, methyl-salicylic acid; SAM, S-adenosylmethionine; SAMT, salicylic acid carboxyl methyltransferase. (**C**) Auxin signaling pathway. ACC, 1-aminocyclopropane-1-carboxylate; ACO, ACC oxidase; ARF, auxin response factor; TIR1, TRANSPORT INHIBITOR RESPONSE 1.

Targets of novel miRNAs from rice

To further understand the biological function of the newly identified miRNAs from rice, we used the degradome sequencing dataset and identified target genes for 6 of the 9 novel miRNAs (Table 2). All these target transcripts have degradome tags by previously described parameters (representative t-plots for some target genes for novel miRNAs are in Supplemental Figure 5B). Unlike conserved miRNAs, the target genes for novel miRNAs were not enriched in transcription factors. Most the miRNA targets for novel miRNAs from rice are involved in biotic stress, whereas other target genes are involved in vesicle transport, cell wall, lipid metabolism and development. The identification of target genes for the newly identified miRNAs provides further evidence that they are *bona fide* miRNAs from rice. Target genes not being detected for the 3 novel miRNAs could be due to the low abundance of the target transcript, inefficient miRNA-directed cleavage, or miRNA-guided translational repression of target genes.

Table 4 Target genes for novel miRNAs from rice identified by degradome analysis. Listed are miRNA/target gene pairs with the target gene classified in categories 0, 1 or 2 in the degradome analysis.

<table>
<tr><th>MicroRNA</th><th>Target gene</th><th>Function of the target gene</th><th>Cleavage position</th><th>Target and miRNA pairing</th></tr>
<tr><td>miR11336-5p</td><td>hydrolase
LOC_Os03g62070</td><td>Cell wall</td><td>1417</td><td>5' ATATATATATGGACAATGCTAGAAAGTCTTAC 3'
||| ||| |||o||||||||||||
3' CTTACACCCGTTGCGATCTTTCAG 5'</td></tr>
<tr><td>miR11336-5p</td><td>disease resistance RPP13-like protein 1 *
LOC_Os01g57270</td><td>Biotic stress</td><td>4181</td><td>5' CCAAAGATTTTAAGATTGCCCATATCCGCTGG 3'
|||||| ||||||||o|| |o
3' AAAATTGTAACGGGTGTAAGT 5'</td></tr>
<tr><td>miR11336-3p</td><td>ankyrin
LOC_Os02g29140</td><td>Biotic stress</td><td>1556</td><td>5' TGTAAGTCATTCTAGCA-TTTCCACATTCATA 3'
|| | |||||||| ||o||| |||
3' TCTGAAAGATCGTAAAGGGTATAA 5'</td></tr>
<tr><td>miR11336-3p</td><td>OsFBDUF13 - F-box and DUF domain containing protein
LOC_Os02g44990</td><td>Unidentified</td><td>1812</td><td>5' TGTAAATCATTTTAGCATTTCCCACATTCATA 3'
| | ||o||||||||||||| |||
3' TCTGAAAGATCGTAAAGGGTATAA 5'</td></tr>
<tr><td>miR11336-3p</td><td>DEFL51 - Defensin
LOC_Os04g15760</td><td>Biotic stress</td><td>1637</td><td>5' ATATGAATGTGGGCAATGCTAGAAAGTCTTAT 3'
||||||||||||o|||||||||||
3' CTTACACCCGTTGCGATCTTTCAG 5'</td></tr>
<tr><td>miR11336-3p</td><td>zinc finger family protein
LOC_Os06g47850</td><td>RNA processing and transcription</td><td>100</td><td>5' TGTAAGTCATTCTAGTATTTCCCACATTCATA 3'
|| | ||||||o|||||||| |||
3' TCTGAAAGATCGTAAAGGGTATAA 5'</td></tr>
<tr><td>miR11336-3p</td><td>terpene synthase
LOC_Os07g11790</td><td>Lipid metabolism</td><td>1809</td><td>5' TTAGCATCAATATGAATGTGAGAAATGCTAGA 3'
| ||||||||||| | |||||
3' GATATACTTACACCCGTTACG 5'</td></tr>
<tr><td>miR11336-3p</td><td>PPR repeat domain containing protein
LOC_Os08g06500</td><td>Biotic stress</td><td>4571</td><td>5' TGAAAGTCATTCTAGCATTTCCCACATTCATA 3'
|| | |||||||||||||||| |||
3' TCTGAAAGATCGTAAAGGGTATAA 5'</td></tr>
<tr><td>miR11336-3p</td><td>dihydroflavonol-4-reductase
LOC_Os08g40440</td><td>Development</td><td>1318</td><td>5' TATAAGTCATTCTATTATTTCCCACATTCATA 3'
|| | ||||| o|||||||| |||
3' TCTGAAAGATCGTAAAGGGTATAA 5'</td></tr>
<tr><td>miR11336-3p</td><td>vesicle-associated membrane protein
LOC_Os12g44250</td><td>Vesicle transport</td><td>877</td><td>5' TGTAAGTCATTCTAGCATTTTCCACATTCATA 3'
|| | |||||||||||o||| |||
3' TCTGAAAGATCGTAAAGGGTATAA 5'</td></tr>
<tr><td>miR11337_3p</td><td>PHD finger protein
LOC_Os05g07040</td><td>Development</td><td>575</td><td>5' TCTGGTTCAAGAC-AATCTAAGGAGAGGGACA 3'
| |||| ||||||| |||| o
3' GCTCTGCTTAGATTGCTCTAT 5'</td></tr>
<tr><td>miR11339-5p</td><td>terpene synthase *
LOC_Os07g11790</td><td>Lipid metabolism</td><td>1809</td><td>5' TTAGCATCAATATGAATGTGAGAAATGCTAGA 3'
| ||||||||||| | |||||
3' GCATTGCCCACATTCATATAG 5'</td></tr>
<tr><td>miR11343-5p</td><td>MLO domain containing protein *
LOC_Os10g39520</td><td>Biotic stress</td><td>1915</td><td>5' TTAACATCAATATGAATGTGGGAAATACTAGA 3'
| ||||||||||||| ||| |
3' GATATACTTACACCCGTTACG 5'</td></tr>
<tr><td>miR11344-3p</td><td>PPR repeat domain containing protein *
LOC_Os08g06500</td><td>Biotic stress</td><td>4573</td><td>5' AAAGTCATTCTAGCATTTCCCACATTCATATT 3'
|| ||o|||| ||||||||||
3' AAAATTGTAACGGGTGTAAGT 5'</td></tr>
<tr><td>miR11342-3p</td><td>transcription factor BIM2
LOC_Os09g29930</td><td>Biotic stress</td><td>381</td><td>5' AGATACTTAGGGAGCTGTTACCTCATAGCGAC 3'
||||| | |o| |||||||o||
3' AATCCAT-GGCCATGGAGTGTC 5'</td></tr>
</table>

Discussion

In this study, we present a comprehensive characterization of rice miRNAs that are regulated by elicitors from the rice blast fungus *M. oryzae*. We identified miRNAs representing 332 and 326 miRNA families in sRNA libraries from rice leaves and roots, respectively. Most contained one or more members with elicitor responsiveness. We previously reported a group of rice miRNAs belonging to 63 miRNA families that respond to treatment with *M. oryzae* elicitors (Campo et al. 2013). Here, in-depth analysis of the miRNA transcriptome validated previous data and significantly extended the list of elicitor-regulated rice miRNAs. The large number of miRNAs showing elicitor responsiveness and their dynamic response to elicitor treatment reflects the complexity of processes that are under miRNA regulation. Presumably, dynamic alterations of miRNA accumulation would allow for a fine-tuning of host gene expression, which would then contribute to maintain timely and appropriate levels of target transcripts in the cell. These findings also support that the rice response to *M. oryzae* elicitors is controlled, at least in part, by miRNA-mediated cleavage of an important number of rice genes. Some of the miRNAs identified in this study may represent rice-specific miRNAs, some having specific functions in PTI responses against the blast fungus. Recently, Li et al.(Li et al. 2013) reported miRNAs that differentially respond to blast infection in resistant and susceptible rice varieties. However, in that study an important number of miRNAs mapped to the *M. oryzae* genome (5%-25% depending on the sRNA library) indicating that the identified miRNAs were of both rice and/or *M. oryzae* origin. Use of fungal elicitors excludes the possibility of miRNAs of fungal origin being in the sRNA sequencing dataset.

Deep sequencing of the sRNA transcriptome also allowed the confident identification of 9 novel miRNAs from rice (miR11336 to miR11344). Moreover, new members of known miRNA families are described. While potentially sacrificing several *bona fide* miRNAs, we used a highly stringent approach to avoid false positives. In this way, we identified both miRNA -5p and -3p sequences with a 2-nt 3’ overhand, a signature of DCL activity, in our sRNA sequencing dataset for all 9 novel miRNAs. Although the newly identified miRNAs were expressed at relatively low levels, Northern blot analysis confirmed the accumulation of 8 novel miRNAs. Furthermore, in this work, we took advantage of the public sRNA high-throughput (HTP) sequencing data of rice. We searched for the sRNA species mapping to the

novel miRNA candidates in rice *DCL1* interference transgenic lines (*dcl1-IR* plants) (Wu et al. 2010). The datasets included GSM520637 prepared from seedlings of rice *DCL1* RNAi transgenic lines and GSM520640 generated from control wild-type plants, all retrieved from GEO (http://www.ncbi.nlm.nih.gov/geo/). Compared to the wild-type plants, the *dcl1-1R* plants accumulated lower levels of miR11336 (both -3p and -5p sequences), miR11337 (-5p sequence), miR11338 (-3p sequence), miR11339 (both -3p and -5p sequences), miR11340 (-3p sequence) and miR11344 (-5p sequence) (Supplemental Figure 8). The dependency on DCL1 for accumulation of these sRNAs further supports that they are *bona fide* novel miRNAs.

Degradome analysis confirmed target genes that are subjected to miRNA-guided regulation in rice, which included conserved and non-conserved targets for known miRNAs. GO analysis of target genes for miRNAs confirmed by degradome analysis revealed enrichment of genes involved in stress responses in elicitor-treated rice tissues. For instance, ROS species are known to be produced under infection conditions, which can rapidly damage biomolecules, so a balance must be maintained between the production and scavenging of ROS. As expected, we found that genes involved in protection against oxidative stress, such as SOD1 and SOD2, glutaredoxin, ascorbate oxidase, or glyoxalase, were among the target genes of elicitor-regulated miRNAs (Table 1). One elicitor-regulated identified was miR398, which targets SOD1 and SOD2. Overexpression of miR398b in rice enhances resistance to *M. oryzae* (Li et al. 2013). Ascorbate oxidase (targeted by miR528) is an apoplastic enzyme that catalyses the first step in degradation of ascorbic acid, thus providing the major redox buffering capacity of the apoplast. Ascorbate oxidase has also been proposed to catalyze the oxidative decarboxylation of auxin, which suggests a role in regulation of auxin levels (Kerk et al. 2000). In tobacco plants, oxidation of apoplastic ascorbic acid has been associated with loss of the auxin response and susceptibility to *P. syringae* (Pignocchi and Foyer 2003). The identified miRNA/target gene pairs involved in protection against oxidative stress might function in a concerted manner to maintain the dynamic balance of ROS levels, thereby maintaining the physiological redox status of the plant cell during elicitor treatment.

Among the confirmed targets for elicitor-regulated miRNAs, we identified components of the plant basal defense response, such as *LTP*, chitinase and PPR-containing protein genes (Cletus et al. 2013; Shah and Zeier 2013; Park et al. 2014). We also validated a miRNA-guided cleavage of rice mannose-binding lectin and remorin genes (targets of miR1439 and miR2925, respectively) (Table 1). Plant mannose-binding lectins are crucial for plant defense

signaling during pathogen attack by recognizing specific carbohydrates on pathogen surfaces (VanDamme et al. 2008). Remorin proteins are found in the plasma membrane in specialized compartments known as membrane rafts, which are platforms for signal transduction during plant–microbe interactions (Jarsch and Ott 2011). Our degradome analysis also identified an important number of transcription factors. Presumably, each transcription factor might further regulate a set of other genes; thus, elicitor-regulated miRNAs might control numerous genes and processes through a complex gene regulation network. In addition, our degradome analysis identified targets for 6 of the 9 novel miRNAs reported in this work. The targets included disease resistance genes (RPP13, MLO), defensin, PPR proteins, ankyrin, and vesicle-associated membrane protein genes.

However, for many miRNAs, we did not identify any target. Taking into account that we limited the search in degradome libraries to categories 0, 1, and 2 (PAREsnip), it is likely that many other miRNA/target gene interactions will be missed. Several possibilities could explain why target genes for miRNAs were not detected. As previously mentioned, some target genes might be difficult to detect because of low abundance or because they guide translational repression of target genes. Alternatively, because we analyzed only two times of elicitor treatment, a time-delay between upregulation of a particular miRNA and target cleavage may occur, as was previously reported for some miRNA/target interactions (Siré et al. 2009).

Although star strands of the duplex have been traditionally considered nonfunctional, evidence here presented supports that the star strand might also have a role in silencing. Thus, we identified miRNA and miRNA* sequences for 19 rice miRNAs for which degradome data confirmed the existence of targets for the two members of the miRNA/miRNA* duplex. A well-known example of a miRNA in which the two sRNAs of the duplex are functional is the Arabidopsis miR393. miR393 targets *TIR1*, whereas miR393* regulates a Golgi-localised SNARE protein (MEMB12) that mediates secretion of PR1 (Zhang et al. 2011). In other studies, miR171* was found to silence the expression of the Arabidopsis *SUVH8* gene, also known as SET DOMAIN GROUP21 (Manavella et al. 2013). Confirmation of target genes for both miRNA and miRNA* of distinct rice miRNAs support the operation of miRNA*-mediated mRNA cleavage mechanisms in rice. Whether the two members of the duplex contribute to PTI in rice remains to be determined.

miRNA-mediated gene regulatory networks in the rice response to fungal elicitors

To shed more light on the functional role of elicitor-regulated miRNAs, we searched for regulatory networks enriched in miRNAs that were supported by the identification of the corresponding target genes by degradome analysis. As previously mentioned, we identified an important number of miRNA/target gene pairs involved in sRNA biogenesis and functioning, namely miRNAs, hc-siRNAs and ta-siRNAs (see Figure 6A). Each pathway contains two or more steps in which a miRNA/target pair participates for generating a specific class of sRNA or accommodation of the sRNA in the RISC silencing complex. The observed elicitor-regulated expression of miR162 and miR168 (targeting DCL1 and AGO1 transcripts, respectively) is consistent with a regulation of the miRNA machinery itself by fungal elicitors. Presumably, an adjustment of miR162 and miR168 levels by elicitors would contribute to maintenance of appropriate levels of DCL1 and AGO1 and, accordingly, miRNA functioning. Supporting this hypothesis, alterations in miR168 and/or AGO1 expression have been described in different plant–pathogen interactions or in response to treatment with elicitors (Peláez and Sanchez 2013; Baldrich et al. 2014; Shen et al. 2014). Also, *dcl1* and *ago1* mutants are compromised in PTI responses or flg22-induced disease resistance in Arabidopsis (Navarro et al. 2008; Li et al. 2010a).

Hc-siRNAs guide DNA methylation at target genomic loci via RNA-directed DNA methylation (RdDM), which reactivates transposons and transcription of silenced genes (Chan et al. 2004). RDR2 and AGO4 are components of the hc-siRNA pathway. Here, we found that *RDR2* and *AGO4* transcripts were regulated by the activity of distinct miRNAs (both miR818 and miR1850 target RDR2, whereas miR1847 targets AGO4). This evidence suggests the existence of miRNA-mediated control of the production and functioning of hc-siRNAs in plants. In other studies, the contribution of RdDM in Arabidopsis antibacterial defense was documented (López et al. 2011). Viral and bacterial infections can also modify hc-siRNAs production and alter DNA methylation (Raja et al. 2008; Dowen et al. 2012; Yu et al. 2013).

Our findings also point to a possible regulation of ta-siRNA production by elicitor-regulated miRNAs. We found regulation of *SGS3* and *AGO1* (by miR393 and miR168, respectively), whose activity is known to be important in the generation of ta-siRNAs (Allen and Howell 2010). That SGS3 is involved in pathogen resistance is further supported by Arabidopsis *sgs3* mutants exhibiting enhanced susceptibility to pathogen infection (Ellendorff et al. 2009).

Overall, profiling of rice miRNAs and their target genes reinforce the existence of self-regulatory mechanisms of the miRNA pathway while revealing a miRNA-mediated regulation on the hc-siRNA and ta-siRNA machinery as part of the rice response to fungal elicitors.

We can draw conclusions regarding miRNA-mediated regulatory networks involved in hormone signaling and crosstalk in hormone signaling. The phytohormones ET, SA, and JA as well as polyamines play an important role in disease resistance, including resistance to *M. oryzae* in rice. The methyl esters of JA and SA (Me-JA, Me-SA) can trigger defense responses in plants (Antico et al. 2012; Shah and Zeier 2013). In this study, we identified an important number of miRNA/target gene pairs that control ET signaling or connect the ET pathway with the Me-JA, Me-SA and polyamine biosynthetic pathways. These pathways are connected at the level of SAM, which serves as a precursor for the production of ET, JA- or SA-methyl derivatives, and polyamines. In particular, distinct SAM-dependent carboxyl methyltransferases use SAM as the methyl donor in the biosynthesis of Me-SA (SAMT, SA carboxylmethyl transferase) or Me-JA (JMT, JA carboxylmethyltransferase). Of note, genes encoding SAMT and JMT were under miRNA regulation (miR5512ab and miR1428bcd, respectively) (see Figure 6B). Moreover, SAM is decarboxylated by the activity of SAM decarboxylase (the first enzyme involved in the production of polyamines), and *SAM decarboxylase* transcripts were also under miRNA regulation (miR2101, see Figure 6B). Thus, we can propose a regulatory mechanism in which distinct miRNA/targets operate for the control of SAM level and SAM distribution to sustain defense-related signaling pathways.

Another network in which several miRNA/target gene pairs participate involves auxin signaling. Degradome analysis revealed target genes that function upstream (auxin perception by F-box auxin receptors) or downstream (ARF6, ARF8, ARF9, ARF10, ARF18, ARF20 and ARF22) of the auxin signaling pathway. These genes are regulated by elicitor-responsive miRNAs. We also revealed a miR5809-guided cleavage of *GH3*, which encodes indole-3-acetic acid (IAA)-amido synthetase. The GH3 enzyme catalyzes the synthesis of IAA-amino acid conjugates, thus providing a mechanism for maintaining auxin homeostasis by conjugating excess IAA to amino acids. Thus, treatment with fungal elicitors may alter the accumulation of miRNAs known to modulate auxin signaling pathways.

In addition, miRNAs are key regulators of auxin response pathways associated with developmental programs in plants (Curaba et al. 2014). Auxin interacts with other phytohormone signaling pathways during plant development. MiRNA-mediated regulation of

auxin signaling contributes to antibacterial resistance in Arabidopsis, as illustrated by regulation of *TIR/AFB2* auxin receptor genes by miR393 (Navarro et al. 2006). In other studies, repression of the auxin response pathway increased the susceptibility to necrotrophic fungi (Llorente et al. 2008). In rice, auxin homeostasis regulates the expression of rice defense genes and resistance to blast fungus (Domingo et al. 2009). In addition to reprogramming host developmental processes, elicitor-regulated accumulation of these miRNAs might well contribute to regulation of defense responses directly or indirectly via cross-talk between auxin and other defense-related hormones.

Hormonal cross-talk has emerged as a major player in regulating tradeoffs between growth and PTI-mediated defense (Huot et al. 2014). The observed connections between ET signaling and the SA and JA signaling pathways via miRNA activities raises some interesting questions regarding the possible role of miRNAs in regulating plant development and defense responses in rice. Because miRNAs provide quantitative regulation of target gene expression rather than on–off regulations, elicitor-mediated regulation of gene expression might help fine-tune host gene expression in reprogramming developmental programs and defense while avoiding the fitness costs associated with the expression of host defense responses. This process would then be part of the adaptive strategy of plants to pathogen infection. A fine-tuned regulation of immune responses, PTI and/or ETI, would avoid negative effects in plant traits of agronomical interest, such as biomass and seed production. The literature contains several examples of miRNAs that regulate growth and development and also mediate plant responses to biotic or abiotic stress (Sunkar et al. 2012). As an example, Arabidopsis miR396 is a developmental regulator in the reprogramming of root cells during cyst nematode infection (Hewezi et al. 2012). Understanding the specific function of rice miRNAs controlling plant responses to pathogen infection and developmental cues will provide powerful tools to optimize the growth–defense balance, which, in turn, will help to improve rice productivity. How plants coordinate the dual function of certain sRNAs in development and defense deserves further investigation.

Regulation of CPuORF-containing genes by elicitor-responsive rice miRNAs

uORFs are *cis*-acting RNA elements involved in translational regulation of the main coding region located downstream of the uORF sequence encoding the conserved short peptide (sPEP). By analogy to miRNAs, which are riboregulators of gene expression, uORF-

encoded short peptides function as "peptoregulators" that mediate translational control of downstream genes (Andrews and Rothnagel 2014). Plant uORFs are classified along evolutionary lines, and for a fairly small fraction, the peptide sequence was conserved during evolution, the so-called CPuORFs. Most genes with CPuORFs have regulatory functions, and transcription factors are over-represented among these genes (Hayden and Jorgensen 2007; Jorgensen and Dorantes-Acosta 2012). We found that two CPuORF-containing bZIP transcription factors from rice, *OsbZIP38* and *OsbZIP27*, are under miRNA regulation (targeted by miR5819 and miR5075, respectively). Of note, the target site of miR5819 locates at the nucleotide sequence encoding the sPEP, as revealed by degradome analysis and further validated by 5'RACE. We present evidence for regulation of the CPuORF7-containing *SAM decarboxylase* gene by miR2101. From these findings, we propose miRNA-guided regulation of CPuORF-containing genes during elicitor treatment. Thus, this regulatory mechanism represents an additional layer of control in the refined regulatory system based on functioning of CPuORF-encoded short peptides.

Certain CPuORF-encoded sPEPs are activated by metabolic signals (i.e., sucrose, polyamines), which offers a path for metabolic control of gene expression (Jorgensen and Dorantes-Acosta 2012). In particular, sucrose can repress translation of *AtbZIP11* and *AtbZIP2* (members of the S1-group of bZIP transcription factors) (Wiese et al. 2014). Indeed, members of the group S of bZIP transcription factors from different species contain a sucrose-regulated CPuORF in their 5' UT region, also known as Sucrose Control-uORFs (SC-uORFs). The SC-uORFs can be found in long (e.g., *AtbZIP11* and *AtbZIP2*) and short versions (e.g., *AtbZIP3*), the later representing the more conserved C-terminal of SC-uORFs that are known to mediate sucrose-induced repression of translation of the *bZIP* gene (Figure 7A). Mutations in the long *AtbZIP11* that creates a shorter conserved uORF (C-terminal conserved amino acids) still allow for sucrose-induced translational control, indicating that the C-terminal amino acids are essential for sucrose regulation (Wiese et al. 2014). Of interest, the two CPuORF-containing transcription factors we identified that are under miRNA regulation (*OsbZIP38* and *OsbZIP27*) contain the short version of the SC-uORF (Figure 7A). This observation suggests sucrose-regulated translational control in the expression of the two rice *bZIP* transcription factors. Sucrose is a signal molecule for activation of plant defense responses (Murillo et al. 2003; Gómez-Ariza et al. 2007; Tauzin and Giardina 2014). *A priori*, the interaction of the miRNA regulation of CPuORF-containing genes and translational control by sucrose would allow these regulatory genes to respond in a

flexible way to rapidly changing stimuli that affect sucrose levels in plants, including pathogen infection. Thus, we propose a model for miR5819 function in regulating *OsbZIP38* expression (Figure 7B). This regulatory network integrates miRNA function and metabolic regulation of gene expression, thereby representing a novel regulatory network potentially involved in plant immune responses. Overall, we provide important clues to further understand the miRNA-mediated and metabolic regulation of CPuORF-containing genes in plants.

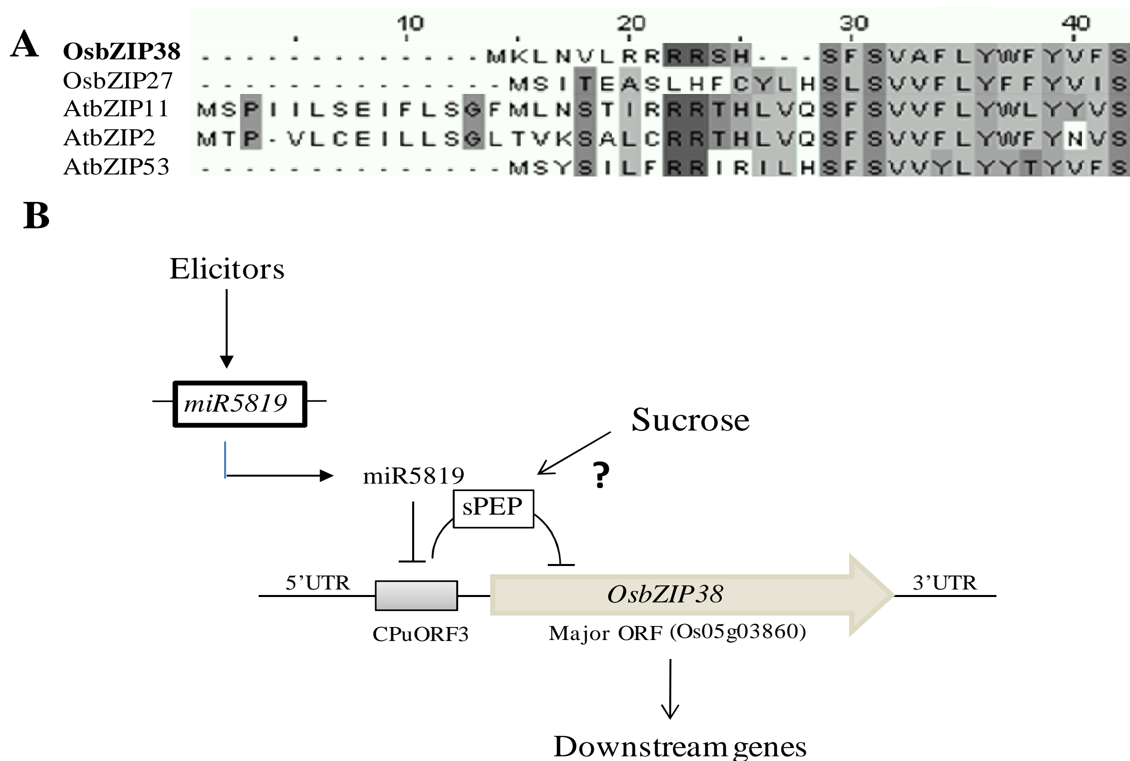


Figure 13: miR5819-mediated regulation of *OsbZIP38* in rice in response to treatment with fungal elicitors. (A) Alignment of the conserved Sucrose Control-uORF (SC-uORF) amino acid sequences present in the 5' UT region of the group S bZIP-type transcription factors in Arabidopsis (*AtbZIP11* and *AtbZIP2*), and rice miRNA-regulated bZIP transcription factors identified in this study (*OsbZIP38*, *OsbZIP27*). Dark and light grey indicate different amino acids. (B) A model depicting the regulation of *OsbZIP38* expression by miR5819 and CPuORFs. Treatment with fungal elicitors regulates miR5819 accumulation, which in turn negatively regulates the accumulation of *OsbZIP38* transcripts. The target site of miR5819 locates at the nucleotide sequence encoding the short peptide (sPEP, encoded by CPuORF3). Sucrose can modulate translation of *AtbZIP11* and *AtbZIP2* (members of the S1-group of bZIP transcription factors) via SC-uORF (Wiese et al. 2004). Whether OsbZIP38 is translationally controlled by sucrose remains to be determined.

To conclude, our results support that miRNAs and their corresponding target genes can be considered an integral part of the rice response to *M. oryzae* elicitors. Disease-resistant rice plants can be obtained by altering the expression of the miRNA or its target gene. Since pathogen attack is one of the primary causes of crop losses worldwide, unravelling the

miRNA-mediated mechanisms underlying pathogen resistance of plants has profound significance.

Materials and Methods

Plant material and elicitor treatment

Rice (*Oryza sativa* japonica cv. Nipponbare) plants were grown at 28º±2ºC under 16-h/8-h light/dark cycles. Elicitors from the *M. oryzae* strain 18.1 were prepared as previously described (Casacuberta et al. 1992) and used at a final concentration of 300 µg/mL. In all experiments, mock treatments were performed. The plant material was harvested at 30 min and 2 h of elicitor treatment. Three biological replicates were analyzed. Each sample represented a pool of approximately 150 rice plants.

Construction of sRNA and degradome libraries and sequencing

Total RNA was extracted from rice tissues with use of TRI Reagent solution (Ambion, Austin, TX, USA). In all, 24 sRNA libraries were prepared by using the TruSeq™ Small RNA kit (Illumina Inc, CA, USA) from leaves and roots that had been treated or not with fungal elicitors (two times of elicitor treatment each tissue, 30 min and 2 h; 3 biological replicates per sample).

Four degradome libraries were prepared from control and elicitor-treated rice leaves (two times of elicitor treatment, 30 min and 2 h) as previously described (German et al. 2009). Small RNA and degradome libraries were individually sequenced on an Illumina Genome Analyzer (HiSeq2000). All the small RNA and degradome sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE66611). Bioinformatic analysis of sequencing data is detailed in Supplemental data. Gene ontology (GO) analysis and GO enrichment of target genes identified in the degradome libraries involved use of MapMan (http://mapman.gabipd.org/web/guest/mapman).

Identification of novel miRNAs

miRDeep-P with default parameters was used to identify novel miRNAs from rice as described (Yang and Li 2011). For this, all the sRNA sequences were mapped to the rice genome (*O. sativa*, version 7.0; http://rice.plantbiology.msu.edu/). Next, genome sequences spanning the putative miRNA, 500 nt upstream and downstream sequences were extracted and used for fold-back secondary structures by use of RNAfold with default parameters (Vienna package 2.1.0; http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Sequences that met the criteria for recognition of candidate miRNA precursors described by Meyers et al. (Meyers et al. 2008) were considered.

Expression analyses

For Northern blot analysis of rice miRNAs, total RNAs were fractionated in a 17.5% denaturing polyacrylamide gel containing 8 M Urea, transferred to nylon membranes (Hybond-N, GE Healthcare, United Kingdom), and probed with [γ^{32}P]ATP-labelled oligonucleotides (Supplemental Table 6). Hybridization signals were detected by use of Phosphorimager (BioRad, CA, USA). Synthetic RNA oligonucleotides were loaded as size markers.

Stem-loop RT-qPCR was used for miRNA expression analysis as described. Further experimental details can be found in Supplemental data.

Modified 5′-RNA ligase-mediated RACE for mapping mRNA cleavage sites

RNA ligase-mediated 5’ RACE was performed as described in Llave et al. 2011 (Llave et al. 2011) with specific primers listed in Supplemental Table 6 and the First-Choice RLM kit (Ambion, Austin, TX, USA). Amplification products were cloned, and at least 15 independent clones were sequenced.

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Authors' Contributions

PB carried out most of the experimental work and data analyses. SC participated in small RNA library preparation. BSS coordinated the design and execution of this study and wrote the manuscript. M-TW, T-TL and Y-ICH participated in degradome analysis. Y-ICH also participated in the design of this study and critically revised the manuscript.

Supplemental Materials

Supplementary Methods. Bioinformatic analysis of sequencing data and Stem-loop RT-PCR.

Supplemental Figure 1. Summary of Illumina sequencing data of small RNAs from rice.

Supplemental Figure 2. Nucleotide sequences of precursors containing novel members of known miRNA families from rice.

Supplemental Figure 3. Nucleotide sequences and chromosomal location of precursors containing novel miRNAs from rice.

Supplemental Figure 4. Elicitor-responsiveness of novel miRNAs from rice.

Supplemental Figure 5. Target plots (t-plots) demonstrating miRNA-mediated transcript cleavage of target transcripts for known miRNAs.

Supplemental Figure 6. Number of target cleavage signals for each miRNA family.

Supplemental Figure 7. Cleavage of CPuORF-containing genes by miRNAs.

Supplemental Figure 8. Accumulation of small RNAs mapping to the novel miRNA candidates in rice *DCL1* RNAi transgenic lines relative to wild-type plants.

Supplemental Table 1. Nucleotide sequences and expression of known miRNAs in rice tissues (leaves, roots) that have been treated, or not, with fungal elicitors.

Supplemental Table 2. Nucleotide sequences and expression of novel miRNAs in rice tissues (leaves, roots) that have been treated, or not, with fungal elicitors.

Supplemental Table 3. Statistics of data generated from sequencing of degradome libraries from control and elicitor-treated leaf libraries.

Supplemental Table 4. Complete list of target genes of known rice miRNAs identified by degradome analysis.

Supplemental Table 5. Target genes that are regulated by more than one miRNA.

Supplemental Table 6. List of oligonucleotides used in this study.

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Chapter III

Genome-wide analysis of polycistronic microRNAs in cultivated and wild rice.

Submitted as: **Genome-wide analysis of polycistronic microRNAs in cultivated and wild rice** to Genome, biology and evolution

Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that direct post-transcriptional gene silencing in eukaryotes. MiRNAs are frequently clustered in the genomes of animals and can be either independently transcribed or simultaneously transcribed into single polycistronic transcripts. In plants, however, only a few miRNA clusters have been described and most of them are generated from independent transcriptional units. Here, a combination of bioinformatic tools and experimental analyses was used for the discovery of new polycistronic miRNAs in rice. A genome wide analysis of clustering patterns of miRNAs in the rice genome revealed 23 loci with the ability to form the typical hairpin structure of miRNA precursors in which two or more mature miRNAs mapped along the same structure. Evidence is presented on the polycistronic nature of 7 miRNA precursors containing homologous or non-homologous miRNA species. By examining the existing data on miRNA profiling, we demonstrated co-expression of individual miRNAs in polycistronic precursors, although a differential accumulation of individual miRNAs derived from a common precursor could also be observed. Polycistronic miRNAs located across different rice chromosomes, except chromosome 12, in both duplicated and non-duplicated regions of the rice genome. Finally, a pattern of conservation in the genome of rice species that have an AA genome was observed for most polycistronic miRNA precursors. The diversity in the organization of *MIR* genes to be produced as polycistrons might provide a versatile mechanism for the control of different biological processes and strength the idea of additional levels of complexity in miRNA functioning in plants.

Introduction

Rice is one of the world's most important food crops and a main food source for more than half of the world population. The genus *Oryza* comprises two cultivated and 22 wild species (http://www.gramene.org). The two cultivated species, *O. sativa* (Asian rice) and *O. glaberrima* (African rice), originated from a common ancestor with AA genome. *O. rufipogon* (perennial species) and *O. nivara* (annual species) have been proposed to be the direct ancestors of *O. sativa* (Sweeney and McCouch 2007; Dogara and Jumare 2014). *O. glaberrima* was independently domesticated from the wild progenitor *O. barthii* after the domestication of Asian rice (Wang et al. 2014)). Furthermore, the cultivated rice varieties belonging to the *O. sativa* group include *japonica* and *indica* subspecies. Genome-sequencing data for a large panel of geographically diverse accessions of the wild rice species *O. rufipogon* and cultivated *indica* and *japonica* varieties was used to construct a model to explain the origin of cultivated rice (Huang et al. 2012). According to this model, *O. sativa japonica* was first domesticated around the middle region of the Pearl River in Southern China, and *O. sativa indica* rice was subsequently developed from crosses between japonica rice and local wild rice as the initial cultivars spread into South East and South Asia. At present, the Asian rice *O. sativa* is cultivated worldwide, while *O. glaberrima*, which is well adapted for cultivation in West Africa, is being gradually replaced by Asian varieties of *O. sativa*.

MicroRNAs (miRNAs) are small non-coding RNAs that emerged as important regulators in post-transcriptional regulation of gene expression (Jones-Rhoades et al. 2006). In plants, many developmental processes are known to be regulated by miRNAs, including organ polarity and morphogenesis, developmental phase transition, floral identity and flower development, shoot and root development and hormone signaling (Aukerman and Sakai 2003; Palatnik et al. 2003; Mallory et al. 2004; Chuck et al. 2007; Chen 2009; Rubio-Somoza and Weigel 2011).MicroRNAs are also associated to the plant response to environmental stress, abiotic and biotic stresses (Chiou et al. 2006; Navarro et al. 2006; Sunkar et al. 2007; Padmanabhan et al. 2009; Jeong and Green 2013; Staiger et al. 2013; Yang and Huang 2014).

Genes encoding miRNAs (*MIR* genes) are transcribed as long primary transcripts (pri-miRNAs) with unique stem-loop structures which are sequentially processed by the RNase III DICER-like (DCL) enzyme, typically DCL1, resulting in a miRNA-5p/miRNA-3p duplex (also named miRNA/miRNA*) (Kurihara and Watanabe 2004; Arikit et al. 2013). The duplex is then exported to the cytoplasm, where the active miRNA sequence is loaded into anArgonaute1 (AGO1)-containing RNA-Induced Silencing Complex (RISC). MiRNAs direct post-

transcriptional gene silencing by guiding cleavage or translational repression of the target transcripts (Llave et al. 2002; Brodersen et al. 2008).

The number of miRNAs has increased substantially in plants and most of the miRNAs that were discovered in early reports are highly conserved throughout the plant kingdom. It is also generally assumed that, after the divergence of dicots and monocots, the number and diversity of miRNAs has changed in a lineage-specific manner (Nozawa et al. 2012). Several mechanisms have been proposed to explain the origin and evolution of plant miRNAs. The prevalent model is that miRNAs arise from inverted duplication of their target genes which would generate a proto-*MIR* gene (Allen et al. 2004; Fahlgren et al. 2007; Axtell and Bowman 2008). Accumulation of mutations would shape the proto-*MIR* into a young *MIR* gene and, eventually, into an ancient *MIR* gene. In addition to direct gene duplication, the spontaneous evolution from random sequences with hairpin-like structures, or derivation from transposable elements, was also proposed to explain the origin of plant miRNAs (Felippes et al. 2008; Piriyapongsa and Jordan 2008). Evolutionary old and conserved miRNAs frequently have more than one gene copy in the genome, whereas new non conserved (or species-specific) miRNAs are usually single copy genes (Allen et al. 2004; Fahlgren et al. 2007).

Processes driving expansion and evolution of miRNAs in plants include whole-genome duplication (polyploidization), duplications of subchromosomal regions (segmental duplications), and local duplications that involve one or two genes (tandem duplications). Segmental and tandem duplication events occurred during evolution in the rice genome (Guyot and Keller 2004; Thiel et al. 2009). Along with this, members of distinct miRNA families have been found to locate in duplicated genomic sequences in rice (i.e. members of the miR395, miR156, and mR169 families) (Jiang et al. 2006). Besides their relevance for expansion and evolution of miRNA family members, duplication events may also lead to diversification in the expression and/or functional properties of miRNAs. For instance, miR164a and miR164b are known to function in lateral root development, whereas miR164c controls petal number in Arabidopsis (Guo et al. 2005; Baker et al. 2005).In other studies, a different expression pattern of miR156 family members in rice tissues, as well as a differential response to treatment with fungal elicitors among members of this family was described (Campo et al. 2013).

MiRNAs can be found either as clusters within a genome region or scattered throughout the genome. Whereas, miRNAs are frequently clustered in the genome of animals, only a few miRNA clusters have been reported in plants (Altuvia et al. 2005; Axtell et al. 2011; Marco et al. 2013). Particularly, clustering of miR166, miR169 and miR395 genes in the genome of several plant species has been reported (Boualem et al. 2008; Zhao et al. 2009; Calviño and Messing 2013; Patanun et al. 2013; Barik et al. 2014). Evidence in animals also support that

clustered miRNAs can be either independently transcribed or simultaneously transcribed into single polycistronic transcripts (Altuvia et al. 2005). These polycistronic miRNAs might contain copies of the same miRNA family member (e.g. homologous miRNAs), or unrelated miRNAs (e.g. non-homologous miRNA clusters) in their precursor structure. Most of the few reported miRNA clusters in plants contain copies of the same conserved miRNAs that are independently transcribed (monocistronic miRNAs).It is also true that plant miRNA precursors are generally much longer and heterogeneous than animal miRNA precursors. Most plant miRNA precursors so far described range from approximately 70bp to several hundreds of bases. It seems then plausible that long miRNA precursors in plants might contain more than one miRNA that can be transcribed as a single transcriptional unit. However, a systematic analysis of polycistronic miRNA precursors in plants is still lacking. In addition to its worldwide agricultural importance, rice represents the model plant for research in monocotyledoneous species which also has a long history of natural selection and selective breeding. Thus, rice provides an excellent system for studies on the evolution and selection of plant miRNAs.

In this work, a genome-wide analysis of clustering patterns of rice miRNAs was carried out. The results presented provide evidence for polycistronic precursors containing homologous and non-homologous miRNAs. Features of rice polycistronic miRNAs including expression patterns of miRNA/target gene pairs are presented. The chromosomal distribution of polycistronic miRNAs in the context of the duplication history of the rice genome is also presented. We discuss the importance of the genomic organization of miRNAs as polycistrons in relation to the co-ordinated regulation of target genes.

Results

Identification of polycistronic miRNAs in rice

Short distances between consecutive MIRNA loci are a hallmark of polycistronic transcription (Altuvia et al. 2005). By using a criterion of 10 Kb distance between to miRNAs, a total of 46 clustered miRNAs are registered in miRBase (release 21) in rice. However, for most of them, it is not known whether they represent polycistronic miRNAs or whether they are independently transcribed. Indeed, clustered *MIR* genes are annotated in miRBase as independent miRNAs.

In this work, we carried out a genome-wide analysis of clustering patterns for *MIRNA* loci representing potential polycistronic miRNA precursors in the rice genome. A stringent criterion of a maximal distance of 3kb between same-strand consecutive miRNAs currently annotated in the miRNA registry (release 21) was used. This analysis scheme was previously used to estimate the fraction of clustered miRNA genes in the human genome (Altuvia et al. 2005). By establishing the threshold of 3kb, we prevent overestimation of the number of clustered miRNAs expressed as single transcriptional units.

For polycistronic miRNAs, the miRNA components are transcribed into a single precursor with multiple miRNA-producing stem loops. We then analyzed the genomic region encompassing each of these miRNA clusters for its ability to form hairpin-forming precursor structures. As a result, we identified 26 loci that contain two or more annotated miRNAs mapping into stem-loop structures of a common precursor (**table 1**). Their length ranged from 134 to 3698 nucleotides.The RNA secondary structures and nucleotide sequence of these putative polycistronic precursors are shown in **supplementary Figure S1 and supplementary Figure S2.** Following the current annotation, the miRNAs originating from opposite arms of the same pre-miRNA are denoted with a -5p or -3p suffix (miRNA or miRNA* species).We named these precursor structures as candidate polycistronic miRNAs.

There were 19 precursor structures containing members of the same family (henceforth homologous miRNAs), such as those comprising miR156, miR166, miR169, miR395, miR399, miR1428, miR1861, miR2118, miR5143 and miR5534 (**table 1**). Three precursors containing 7, 6 and 4 members of the miR395 family, respectively, were also identified which is consistent with previous reports indicating

Table 5. MiRNA precursor structures containing two or more miRNAs in rice.

	miRNA 1	miRNA 2	miRNA 3	miRNA 4	miRNA 5	miRNA 6	miRNA 7	Chr.	Start	End	Length (nt)	Consevation
Homologous miRNAs	miR156c	miR156b						1	4665975	4666516	541	Sb - Gm
	miR166k	**miR166h**						2	32435003	32435292	289	Sb - Gm - Zm - At - Mt
	miR169j	miR169k						9	19788861	19792288	3427	Sb - Gm - Zm - At - Mt
	miR169m	miR169l	miR169q					8	26813897	26817595	3698	Sb - Gm - Zm - At - Mt
	miR395a	miR395b	miR395c	miR395d	miR395e	miR395f	miR395g	4	31804633	31805635	1002	Sb - Gm - Zm - At - Mt
	miR395h	miR395i	miR395j	miR395k	miR395y	miR395l		8	3299144	3300090	946	Sb - Gm - Zm - At - Mt
	miR395t	miR395u	miR395v	miR395w				9	6606291	6607495	1204	Sb - Gm - Zm - At - Mt
	miR399c	miR399h						5	26305938	26308034	2096	Sb - Zm - At - Mt
	miR399e	miR399a						1	30477612	30478784	1172	Sb - Zm - At - Mt
	miR1428e	**miR1428d**						3	23076870	23077391	521	-
	miR1861b	**miR1861c**						2	20715962	20716280	318	-
	miR1861d	miR1861e						4	6536337	6536647	310	-
	miR1861f	miR1861g						5	18668398	18668717	319	-
	miR1861h	miR1861p						6	27238190	27238501	311	-
	miR1861k	miR1861j						8	15132529	15132836	307	-
	miR1861l	miR1861m						9	13532426	13532744	318	-
	miR2118p	miR2118r	miR2118q					11	7807433	7810885	3452	Zm - Mt
	miR5143b	miR5143a						1	8415808	8417315	1507	-
	miR5534a	miR5534b						11	6404295	6404519	224	-
Non-homologous miRNAs	**miR1423**	**miR1868**						4	19715117	19715616	499	-
	miR1876	**miR1862d**						10	4833365	4833760	395	-
	miR5147	**miR437**						2	17049859	17050548	689	-
	miR6255	**miR6253**						7	25018786	25019092	306	-
Mis-annotated miRNAs	miR1440-5p/miR1440-3p(1)							9	5980506	5980694	188	-
	miR2275-5p/miR2275-3p(2)							8	1202490	1205135	2645	-
	miR5512-5p/miR5512-3p(3)							4	18386976	18387110	134	-

miRNA precursor structures containing two or more miRNAs in rice. The criterion of a 3 kb distance between consecutive miRNAs was used to identify candidate polycistronic miRNAs. This analysis was based on miRNAs annotated in the miRBase registry (release 21). As for miR1861, a precursor containing miR1861h-1861p was identified of which miR1861p is not registered in the current version of miRBase (Baldrich et al., 2015).The precursor structures and nucleotide sequences are presented in **supplementary Figure 1 and supplementary Figure 2**, respectively. In bold are polycistronic miRNAs experimentally validated in this study by RT-PCR. The chromosomal coordinates of the precursor comprising two or more miRNAs (start and end) are indicated. Conservation among different plant species is shown (At, *Arabidopsis thaliana*; Mt, *Medicago truncatula*; Gm, *Glycine max*; Sb, *Sorghum bicolor*; Zm, *Zea mays*). (1) These sequences are annotated in miRBase as miR1440b and miR1440a. (2) These sequences are annotated in miRBase as mR2275b and miR2275a. (3)These sequences are annotated in miRBase as miR5512a and miR5512b.

that miR395 is organized into compact clusters in plant species, including rice (Guddeti et al. 2005; Merchan et al. 2009). Furthermore, six precursor structures each one containing two members of the miR1861 family were identified. They were: miR1861b-1861c, miR1861d-1861e, miR1861f-1861g, miR1861h-1861p (miR1861p is a new member of this family, reported by Baldrich et al. 2015), miR1861k-1861j and miR1861l-1861m. A more detailed

analysis of miR1861 precursor structures is presented below. Evidence on the polycistronic nature for 3 out of the 19 homologous polycistronic miRNAs identified in this study was previously reported (e.g. miR166k-166h, miR1428e-1428d, miR1861b-1861c) (Zhu et al. 2008; Barik et al. 2014).

Additionally, we identified four candidate polycistronic miRNAs encoding miRNAs that belong to different families (henceforth non-homologous miRNAs). They were: miR1423-1868, miR1876-1862d, miR5147-437, and miR6255-6253 (**table 1**).

During the course of this study, we also noticed that there were three precursors, each of them comprising two miRNA species which are annotated in miRBase as two different family members of the miR1440, miR2275 and miR5512 families (e.g. “a” and “b” members in each family). The two family members were separated by 187 (miR1440), 2386 (miR2275) and 133 (miR5512) nucleotides (**table 1**). Interestingly, folding analysis revealed that the two miRNA species of the corresponding family mapped opposite to each other in the same precursor structure, having the characteristic 2-nt 3’-overhangs, a signature of DICER cleavage for miR/miR* duplexes (Meyers et al. 2008; Kozomara and Griffiths-Jones 2014). Details on their structure and nucleotide sequences are presented in **supplementary Figure S3 and supplementary Figure S4**, respectively). The miRNA annotation for these particular miRNAs is therefore likely to be incorrect, these sequences representing the miRNA-5p/miRNA-3p species of the corresponding miRNA that can no longer be considered as different family members. These precursors were not considered for further analysis in this work. In this way, 23 candidate polycistronic miRNAs were further investigated.

We searched for conservation patterns of candidate polycistronic miRNAs in other plant species, both monocotyledonous (O. *sativa, Zea mays, Sorghum bicolor*) and dicotyledonous species (*Arabidopsis thaliana, Medicago truncatula, Glycine max*). The same criterion of 3kb as the maximal distance between two miRNAs was used for this analysis. Precursors containing miRNA sequences belonging to the miR156, miR166, miR169, miR395, miR399 and miR2118 families were identified in the genome of one or another species, whereas 13 out of the 23 candidate polycistronic miRNAs were found only in rice, suggesting that these precursors might be rice-specific (**table 1**). In particular, the 4 precursors containing non-homologous sequences were present only in the rice genome.

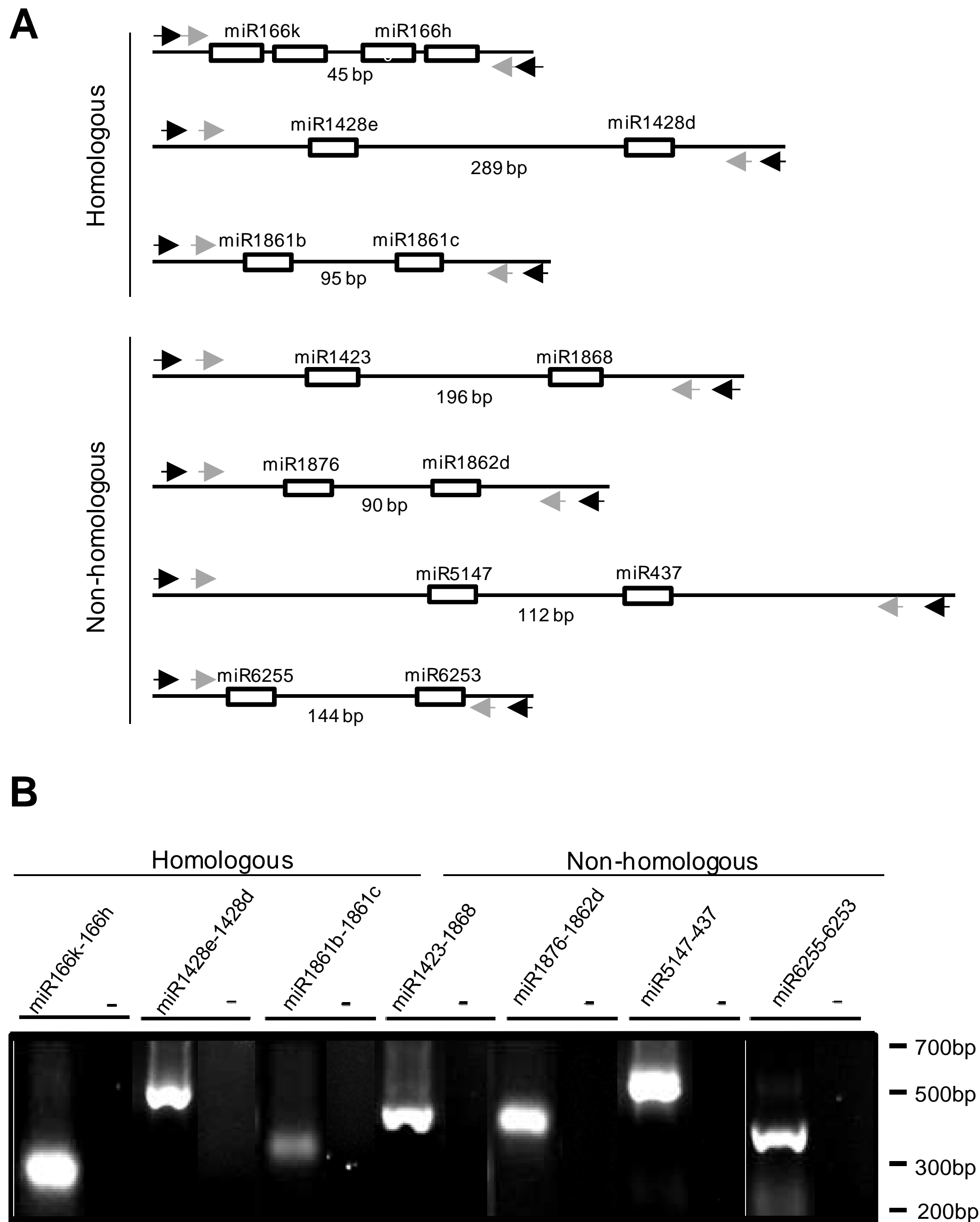


Figure 14. Experimental validation of polycistronic miRNAs in rice. (A) Schematic representation of polycistronic miRNAs showing the positions of mature miRNAs in each precursor (boxes) and distances between them. Arrows indicate the position of primers used for nested RT-PCR in B. (B) Detection of polycistronic miRNA transcripts as revealed by nested RT-PCR. Total RNAs were extracted from leaves of 3 weeks-old rice seedlings, subjected to DNase treatment and used for cDNA synthesis. (-), negative controls (samples in which no reverse transcriptase was added).

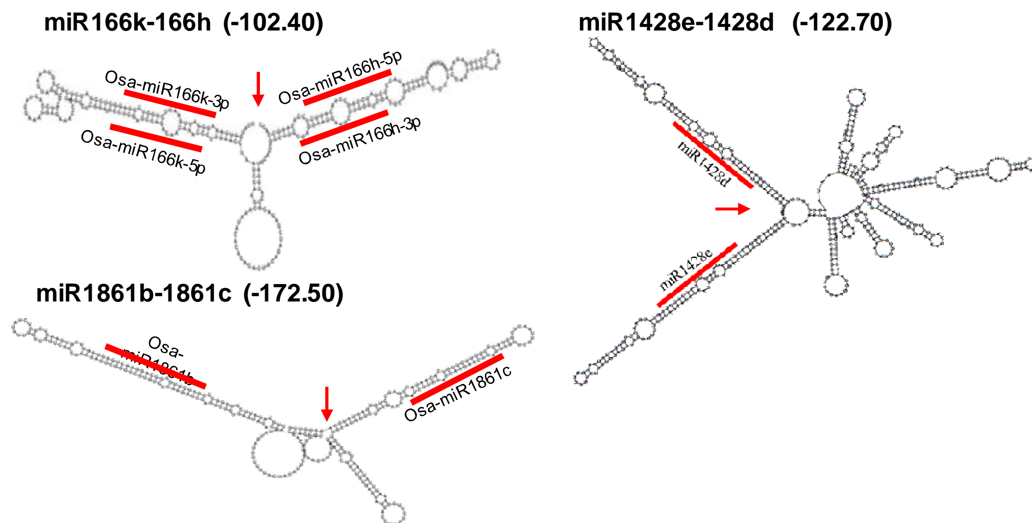
Experimental validation of rice polycistronic miRNAs

As previously mentioned, clustered miRNAs can be either independently transcribed (monocistronic miRNAs) or simultaneously transcribed from a common precursor transcript (polycistronic miRNAs). In this work, we investigated whether candidate polycistronic miRNAs identified in the rice genome are transcribed as polycistronic units. For this, total RNA was isolated from leaves of 3 week-old rice plants. Nested reverse transcriptase-polymerase chain reaction (RT-PCR) and subsequent sequencing of the PCR products confirmed the specific amplification of transcripts for 7 miRNA precursors, each one comprising the expected mature miRNAs (**Figure 1A, B**). These findings support that these miRNA precursors are transcribed as single transcriptional units. Among the experimentally validated polycistronic miRNAs there were homologous (miR166k-166h, miR1428e-1428d, miR1861b-1861c) and non-homologous (miR1423-1868, miR1876-1862d, miR5147-437, miR6255-6253) polycistronic miRNAs. The distances separating mature miRNAs in these precursors ranged from 45 nucleotides to 289 bp **(Figure 1A).** The RNA secondary structure of the various polycistronic miRNAs validated in this study is presented in **Figure 2.**

Concerning miR1861 precursors, the polycistronic nature of only one precursor (miR1861b-1861c) could be validated. Difficulties in designing precursor-specific primer pairs to discriminate among the other miR1861 precursors did not allow us to examine whether they are also transcribed as polycistrons. On the other hand, we were unsuccessful in detecting transcripts for the remaining candidate polycistronic miRNAs. Failure to detect these transcripts could be due to the complex secondary structure of these precursors, low level expression and/or spatiotemporal expression pattern in rice tissues. Further studies are needed to determine whether these candidate miRNA precursors represent true polycistronic miRNAs.

Together, these results support the existence of polycistronic miRNAs in rice comprising homologous or non-homologous sequences while providing evidence for the polycistronic nature of miRNAs that are currently described as clustered miRNAs in the miRBase registry.

A Homologous polycistronic miRNAs



B Non-homologous polycistronic miRNAs

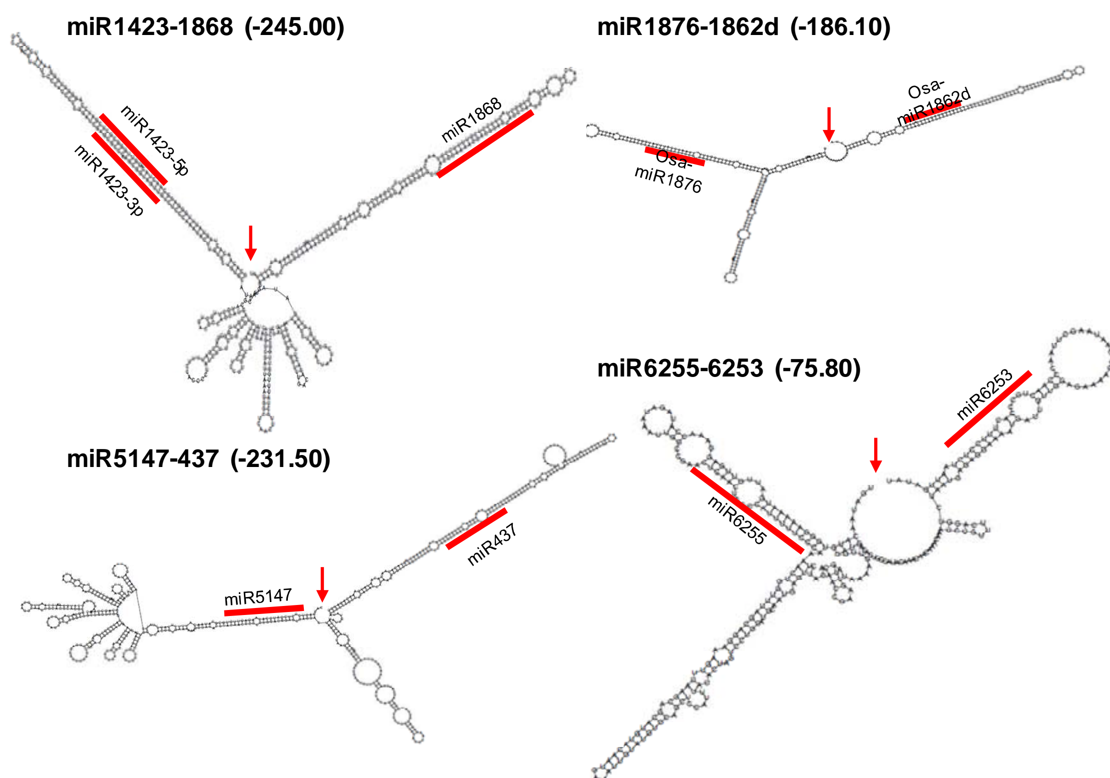


Figure 15. Secondary structures of polycistronic miRNAs that have been experimentally validated in this work. (A) Homologous polycistronic miRNAs. (B) non-homologous polycistronic miRNAs. Their nucleotide sequences are presented in supplementary Figure S2. Mature miRNA sequences are indicated with a black line. Minimal Free Energy (MFE) for each structure indicated in parenthesis.

Polycistronic precursors containing miR1861 family members

In this study, precursors containing members of the rice miR1861 family were examined in more detail. As it is shown in **table 1**, 12 members of the miR1861 family were found to be encoded by pairs in 6 different precursor structures. A comparison of the precursor sequences corresponding to the 6 polycistronic precursors revealed significant similarity among them (**supplementary Figure S5**). Of them, RT-PCR experiments confirmed that miR1861b-1861c is indeed a polycistronic miRNA (**Figure 1**).

To further explore the evolutionary history and relationship among the 6 polycistronic miR1861 precursors, a Neighbour-Joining tree was constructed (**Figure 3**). According to the phylogenetic tree, the miR1861h-1861p and miR1861k-1861j were very closely related, indicating their origin through recent duplications without major changes in the sequences. Moreover, miR1861b-1861c and miR1861f-1861g clustered together, whereas miR1861l-1861m diverged from the other polycistronic miR1861 precursors. It can be hypothesized that duplication of a founding *MIR1861* gene gave rise to an ancestor polycistronic miR1861 precursor containing two miR1861 mature sequences. Subsequent duplication events and diversification from the initial polycistronic precursor would explain the presence of multiple loci for polycistronic *MIR1861* genes, each precursor containing 2 members of the miR1861 family.

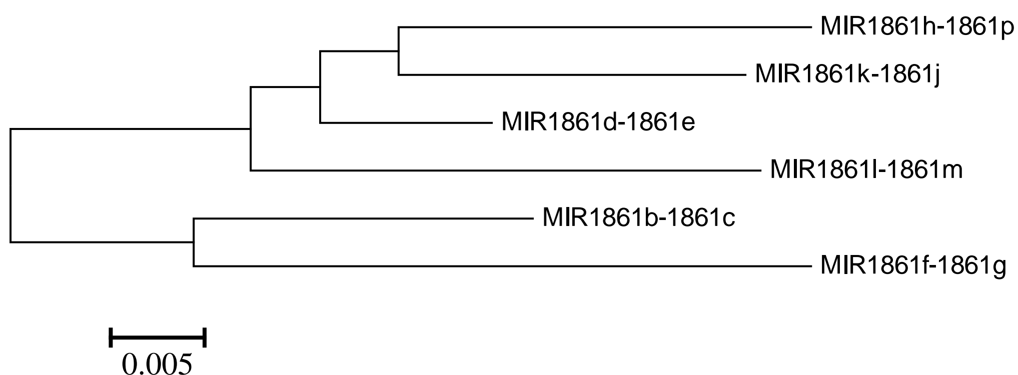


Figure 16. Phylogenetic tree constructed with polycistronic miR1861 precursors using the Neighbour-joining method (Saitou and Nai 1987). Numbers along each branch indicate estimated genetic distance.

Expression of rice polycistronic miRNAs

The co-expression of clustered miRNAs has been used as evidence that they derive from a common primary transcript in eukaryotes (Bartel 2004). However, it is still unclear what determines the relative abundance of individual miRNA components produced by a polycistronic miRNA precursor.

To explore the potential for co-expression of miRNAs contained in polycistronic precursors we examined the existing data on deep sequencing of small RNA libraries from rice (Baldrich et al., 2015). The data sets were all retrieved from the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (*NCBI*) (http://www.ncbi.nlm.nih.gov/geo/;for accession numbers, see Material and Methods). The miRNA expression data corresponded to leaf and root tissues of 3 week-old rice plants (Baldrich et al., 2015). MiRNA species with 50 or more reads in the Illumina sequencing data were considered for this analysis. Small RNA sequencing data was then interrogated for expression of miRNAs contained in the 7 polycistronic miRNAs that have been experimentally validated in this study, both homologous and non-homologous polycistronic miRNAs. As it is shown in **Figure 4A**, pairs of mature miRNAs generated by miR166k-miR166h, miR1423-1868, and miR1876-1862d were found to be co-expressed in both tissues, leaves and roots. Of the two miRNAs encoded by the miR1423-1868 and miR1876-1862d precursors, the miRNAs species located in the 5’ region (e.g. miR1423 and miR1876) accumulated at a lower level compared to the miRNAs located in the 3’ region of these precursors. Additionally, in the case of miR5147-437, the mature miRNA species located in the 5’ region of this polycistron (e.g. miR5147) was barely detected in one or another tissue (**Figure 4A**).

Also, taking advantage of the available information on miRNA expression in rice tissues in response to treatment with fungal elicitors, we examined whether the expression of miRNAs encoded in each polycistronic precursor is altered in the same direction in response to elicitor treatment. The datasets were all retrieved from GEO and cited in Material and Methods. This analysis revealed that mature miRNAs encoded by miR166k-miR166h, miR1423-1868, and miR1876-1862d had the same trend in their response to elicitors, further supporting that they are co-expressed (**Figure 4B**). Of note, a different response to elicitors could be observed at one or another tissue for miRNA species encoded in these precursors (i.e. miR166k-166h was up-regulated in leaves and down-regulated in roots) (**Figure 4B**). The same opposite response was also observed for miR1423-1868 and miR1876-1862d. It can then be hypothesised that a differential regulation of polycistronic miRNA expression might occur in the two rice tissues upon treatment with fungal elicitors. Alternatively, despite their co-transcription as polycistronic precursors, post-transcriptional regulation mechanisms might also result in a

differential accumulation of mature miRNAs derived from these polycistrons in rice tissues (i.e. different stability and turnover of mature miRNAs). Further studies are needed to clarify this aspect.

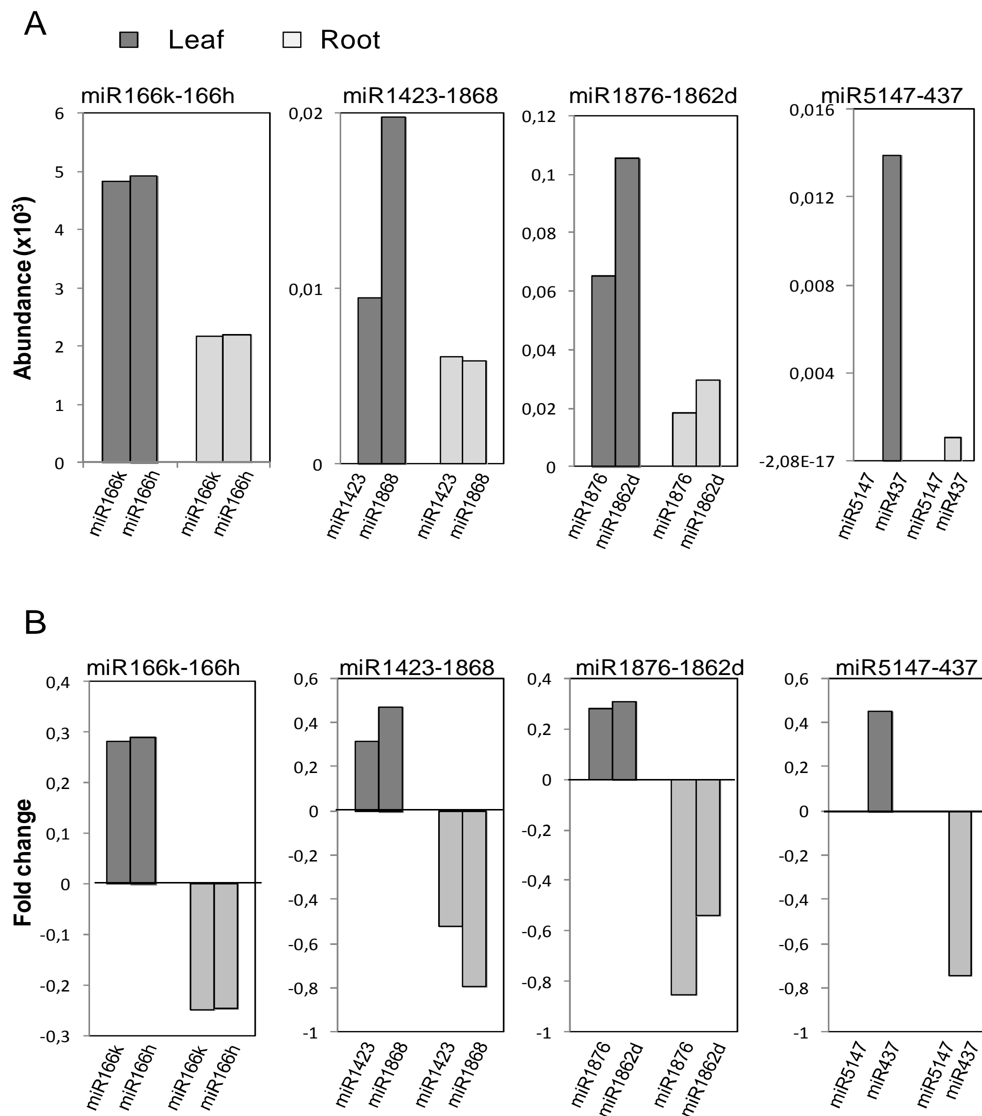


Figure 17. Co-expression of miRNAs produced by polycistronic precursors in rice. Histograms indicate the expression of mature miRNAs encoded in each polycistronic precursor in leaves (dark grey) or roots (light grey). Expression data was retrieved from the Solexa/Illumina sequencing data set deposited in GEO (GSE66611) (Baldrich et al., 2015). Only miRNAs having ≥ 50 reads in Illumina sequencing data of small RNA libraries were considered. In the case of the miR166k-166h precursor, the functional strand of the miRNA-5p/miRNA-3p duplex was examined (e.g. miR166k-3p and miR166h-3p). (A) Expression of miRNAs encoded by polycistronic precursors in leaves and roots. For each miRNA, the number of reads in each tissue was normalized to the total count of reads in the corresponding small RNA library. (B) Elicitor-responsiveness of miRNAs generated by polycistronic precursors. Expression data correspond to tissues, leaves or roots, that have been treatedwith elicitors from the rice blast fungus *Magnaporthe oryzae* for 30 min, relative to control, non-treated tissues (fold change = elicitor *vs.* control).

Target genes for polycistronic rice miRNAs

Target genes for conserved miRNAs are well documented, and in many cases, miRNA/target gene interactions have been functionally characterized. Less information is currently available on target genes for non-conserved miRNAs, including rice-specific miRNAs. Advances in high-throughput sequencing and degradome analysis provided the opportunity to identify miRNA targets in plant species at a genome-wide scale (Addo-quaye et al. 2009; German et al. 2009). Under this scenario, we investigated the target genes for mature miRNAs encoded by polycistronic miRNAs using degradome sequencing data from rice.The degradome data sets were retrieved from the NCBI-GEO database (accession number GSE66611) and corresponded degradomes from leaves of 3 week-old rice plants, as reported by Baldrich et al. (2015). Target genes for miRNAs encoded in homologous and non-homologous polycistronic miRNAs were identified (**table 2**).

Consistent with previous findings, degradome analysis or rice leaves confirmed cleavage of target genes for conserved miRNAs that are encoded by polycistrons (**table 2).** This is the case for the target genes of miR156, miR166 and miR169 (e.g. OsSPL, OsBH and NF-Y transcription factors) which are known be involved in developmental processes and hormone regulation, some of them being also involved in the plant response to abiotic stress (Jones-Rhoades et al. 2006; Mallory and Vaucheret 2006; Wu et al. 2009; Li et al. 2010; Zhou et al. 2010; Cui et al. 2014).

As for miR395-containing polycistrons, three polycistronic precursors containing 4, 6 or 7 miR395 species, respectively, were identified in the rice genome (**table 1**). miR395 is a conserved miRNA that targets ATP sulfurylases in Arabidopsis plants, these enzymes being involved in sulfate uptake and assimilation (Liang et al. 2010; Liang and Yu 2010). Consistent with this, degradome analysis of rice leaves identified ATP sulfurylase as the target gene for mature miR395 species produced by the various miR395 polycistrons (**table 2**). In addition to ATP sulfurylases, degradome analysis also revealed other target genes for miR395 sequences in these polycistrons, which varied depending on the miRNA precursor. For instance, miRNA species in the miR395a-395b-395c-395d-395e-395f-395g precursor were found to cleave uridine ribohydrolase, whereas miR395v in the miR395t-395u-395v-395w cleaved Uridine ribohydrolase and protein kinase transcripts (**table 2**). In the case of the miR395h-395i-395j-395k-395y-395l precursor, one of the members of this polycistron is predicted to target cytochrome b5-like Heme/Steroid binding domain containing protein (**table 2**). In

Table 2. Targets for mature miRNAs encoded by polycistronic miRNAs.

	Polycistron	miRNA	Target	Cleavage site (1)	Biological process	Function	Reference
Homologous miRNAs	miR156c-156b	miR156b-5p	**SBP domain containing protein OsSPL2 (Os02g04680)**	1163			
		miR156c-5p	**OsSPL3 (Os01g69830)**	1975			
			OsSPL4 (Os02g07780)	2231			
			OsSPL11 (Os06g49010)	864			
			OsSPL12 (Os06g45310)	1504	Development	Transcription factor	Wu et al. 2009
			OsSPL13 (Os07g32170)	674			
			OsSPL14 (Os08g39890)	1002			
			OsSPL17 (Os09g31438)	819			
			OsSPL18 (Os09g32944)	1044			
			OsSPL19 (Os11g30370)	1101			
			O-methyltransferase (Os12g25490)	264	Metabolism phenylpropanoids	Transferase activity	Baldrich et al. 2015
			DUF260 domain containing protein (Os12g01550)	638	Development	Transcription regulation	
	miR166k-166h	**miR166h**	**HD-ZIP (OsHB1) (Os03g01890)**	1100			
			HD-ZIP (OsHB2) (Os10g33960)	935			Li et al. 2010
			HD-ZIP (OsHB3) (Os12g41860)	888	Development-auxin signaling	Transcription factor	
			HD-ZIP (OsHB4) (Os03g43930)	966			
		miR166k	homeobox associated leucine zipper (Os10g23090)	180			
	miR169j-169k	miR169j	no target				
		miR169k	no target				
	miR169m-169l-169q	miR169m	**nuclear transcription factor Y subunit (NF-YA) (Os02g53620)**	999			
		miR169l	**NF-YA (Os03g07880)**	855			
		miR169q	**NF-YA (Os03g29760)**	1295	Development - Abiotic stress	Transcription factor	Zhou et al. 2010
			NF-YA (Os03g44540)	1729			
			NF-YA (Os03g48970)	1247			
			NF-YA (Os07g41720)	1180			
			NF-YA (Os12g42400)	1267			
	miR395a-395b-395c-395d-395e-395f-395g	miR395a	**Uridine ribohydrolase (Os09g39440)**	1105	Metabolism - Nucleotide degradation	Hydrolase activity	Baldrich et al. 2015
	miR395h-395i-395j-395k-395y-395l	miR395b	ATP sulfurylase (Os03g53230)		Nutrient stress adaptation	Sulfate assimilation	Li et al. 2010
		miR395h	**cytochrome b5-like Heme/Steroid binding domain containing protein (Os10g35870)**				
		miR395i					
		miR395j		663	Signalling	Oxidoreductase (redox ascorbate & glutatione)	Zhou et al. 2010
		miR395k					
		miR395y					
		miR395l					
		miR395y	ATP sulfurylase (Os03g53230)		Nutrient stress adaptation	Sulfate assimilation	Li et al. 2010
	miR395t-395u-395v-395w	miR395v	**Protein kinase (PVPK-1) (Os10g41290)**	1981	Protein synthesis & degradation	protein serine/threonine kinase	Baldrich et al. 2015
			Uridine ribohydrolase (Os09g39440)	1105	Nucleotide metabolism	nucleotide degradation	
			ATP sulfurylase (Os03g53230)		Nutrient stress adaptation	Sulfate assimilation	Li et al. 2010
		miR395tuvw	pentatricopeptide (Os05g40950)		Protein synthesis	Structural constituent of ribosome	
	miR399e-399a	miR399a	**ubiquitin conjugating enzyme protein (Os05g48390)**				
		miR399e				Aminoacid ligase activity	
				915	Nutrient stress adaptation	protein degradation	
	miR399c-399h	miR399c				Phosphate homeostasis	
		miR399h					
	miR1428e-1428d	**miR1428d**	**expressed protein (Os03g08970)**	1132	Unidentified		
			SAM dependent carboxyl methyltransferase (Os06g22440)	814	Hormone metabolism & signaling	Salicilic acid signalling	
		miR1428e	**DUF584 domain containing protein (Os04g45834)**	1022	Unidentified		Baldrich et al. 2015
			expressed protein (Os03g08970)	1132	Unidentified		

Table 2 (continued). Targets for mature miRNAs encoded by polycistronic miRNAs.

	Polycistron	miRNA	Target	Cleavage site (1)	Biiological process	Function	Referecnce
Homologous miRNAs	**miR1861b-1861c**	**miR1861c**	**glyoxalase family protein (Os08g09250)**	500	Oxidative stress	Detoxyfication of methylglyoxal & recycling of reduced glutathione (GSH)	
			bZIP transcription factor (Os06g10880)	1381	Hormone metabolism & signaling Abiotic stress	Transcription factor	Baldrich et al. 2015
			plus-3 domain containing protein (Os01g56780)	2312	RNA processing & transcription	Transcription factor - growth	
		miR1861b	**glyoxalase family protein (Os08g09250)**	500	Stress - Oxidative stress	Detoxyfication of methylglyoxal & recycling of reduced glutathione (GSH)	
			expansin precursor (Os10g40730)		Cell wall modification	cell wall - promotionof cell growth	
	miR1861d-1861e	miR1861d miR1861e	No target				
	miR1861f-1861g	miR1861f					
		miR1861g	**B-box zinc finger family protein (OsBBX4) (Os02g39360)**	626	Flowering & Abiotic stress	Transcription factor	Huang et al. 2012
	miR1861h-1861p	miR1861h	**B-box zinc finger family protein (OsBBX4) (Os02g39360)**	626	Flowering & Abiotic stress	Transcription factor	Baldrich et al. 2015
		miR1861p	No target				
	miR1861k-1861j	miR1861k	No target				
		miR1861j	**B-box zinc finger family protein (OsBBX4) (Os02g39360)**	626	Flowering & Abiotic stress	Transcription factor	Baldrich et al. 2015
	miR1861l-1861m	miR1861l	**chlorophyll A-B binding protein (Os07g38960)**	503	Light		
		miR1861m	No target				
	miR2118p-2118r-2118q	miR2118p miR2118r	Disease resistance gene (CC-NBS-LRR)		Biotic stress		Shivapresad et al. 2012
	miR5143b-5143a	miR5143a	Phosphoinositide phosphatase SAC4 (Os10g25180)		Metabolism-Signaling	Metabolism of phosphoinositides	
		miR5143b	cytochrome P450 (Os03g04530)		Metabolism	Oxydation reduction process	
	miR5534a-5534b	miR5534a	**expressed protein**				Baldrich et al. 2015
		miR5534b	sucrose-phosphate synthase (Os02g09170)		Carbohydrate methabolism	Major CHO metaboñlism synthesis	
Non-homologous miRNAs	**miR1423-1868**	**miR1423-5p**	**EF h& family protein (Os06g51250)**	3813	Signaling	Calcium signaling	Baldrich et al. 2015
		miR1423-3p	harpin-induced protein 1 domain containing protein (Os04g58860)		Biotic stress	Hypersensitive Response	
			protein kinase domain containing protein (Os01g59560)		Signaling	Serine/treonine phosphotransferase	
		miR1868	No target				
	miR1876-1862d	**miR1876**	pattern formation protein EMB30 (Os04g02690)		Development - Cell division	similar to the yeast SEC7 protein	
		miR1862d	**F-box domain containing protein OsFBK14 (Os04g31120)**	1906	Protein synthesis & degradation	Protein synthesis inhibition	
			ribosome inactivating protein (Os01g06740)	1182			
	miR5147-437	**miR5147**	**protein binding protein (Os01g34780)**	2006	Unidentified		Baldrich et al. 2015
		miR437	**ethylene-responsive transcription factor TINY (Os02g13710)**	894	RNA processing & transcription	Transcription facor	
	miR6255-6253	**miR6255**	**expressed protein (Os01g67370)**	2194	Unidentified		
		miR6253	**MYB family TF (Os08g06110)**	797	Nutrient stess adaptation	Transcription factor - potassium deprivation	

Targets for mature miRNAs encoded by polycistronic miRNAs. In bold, target genes that have been experimentally validated by degradome analysis by Baldrich et al (2015). Evidence for miRNA-guided cleavage of the indicated targets can be found at the indicated references. The remaining target genes have been predicted. (1) Cleavage sites were validated using degradome analysis or 5' RACE (rapid amplification of cDNA ends).

plants, the family of cytochrome b5-like proteins includes the cytochrome b_5 itself and nitrate reductases, these enzymes being involved in nitrate assimilation and nitric oxide production (Horchani et al. 2011).

MiR399 is also a conserved miRNA in plants with a conserved target gene. Two polycistrons encoding pairs of miR399 sequences each were identified in the rice genome. MiR399 cleaves transcripts of a ubiquitin-conjugating E2 enzyme involved in the control of phosphate homeostasis (PHO2) (Fujii et al. 2005; Chiou et al. 2006; Kawashima et al. 2009) (**table 2**). Other target genes for miRNAs encoded by homologous polycistronic precursors were those involved in hormone signaling, such as SAM dependent carboxylmethyltransferase (targeted by miRNAs in miR1428e-1428d) (**table 2**).

Concerning miR1861polycistrons, different target genes were identified for individual miRNAs contained in these polycistrons. Particularly, glyoxalase transcripts were found to be targeted by miRNAs encoded by the miR1861b-1861c precursor (**Table 2**). Glyoxalases play an important role in oxidative stress tolerance (Kaur et al. 2014) by recycling reduced glutathione, thereby maintaining glutathione homeostasis (glutathione-dependent glyoxalase detoxification system). Furthermore, cleavage of bZIP transcription factor and plus-3 domain containing protein transcripts, guided by miR1861c (also derived from the miR1861b-1861c precursor) were identified (**table 2**). On the other hand, a B-box zinc finger family protein gene was found to be cleaved by one of the two miR1861 species contained in three different polycistrons (miR1861g in miR1861f-1861g, miR1861h in miR1861h-1861p, and miR1861j in miR1861k-1861j). Finally, a chlorophyll A-B binding protein gene was found to be targeted by miR1861l derived from the miR1861l-1861m precursor. From these results, it appears that mature miRNAs contained in miR1861 polycistronic precursors share common target genes while some of them also have specific target genes depending on the polycistronic miR1861 precursor.

As for the validated target genes for miRNAs encoded by non-homologous polycistrons, we noticed that they were involved in a range of processes, such as calcium signalling (EF hand family protein targeted by miR1423, miR1423-1868 precursor), protein synthesis and degradation (OsFBK14 and Ribosome Inactivating Protein, targeted by miR1862d, miR1876-1862d precursor), and transcriptional control of gene expression (Ethylene-responsive transcription factor TINY, targeted by miR437, miR5147-437 precursor; MYB transcription factor, targeted by miR6253, miR6255-6253 precursor) (**table 2**). Finally, no function has been assigned for the target genes of distinct miRNAs in non-homologous polycistrons, whereas in other cases no target gene could be identified for these miRNAs.

These findings illustrate the diversity of target genes that are subjected to regulation by miRNAs in polycistronic precursors in rice. The simultaneous production of individual miRNAs

encoded in polycistrons with the ability to target genes involved in different processes expands the gene regulatory capacity of this class of riboregulators which, in turn, might contribute jointly to the control of biological processes in plants.

Chromosomal mapping of rice polycistronic miRNAs

In this work, we examined the chromosomal distribution of rice polycistronic miRNAs. Except chromosome 12, the various polycistronic miRNAs identified in this work distributed among the different rice chromosomes.

Taking advantage of the known duplication history of the rice genome (Guyot and Keller 2004; Yu et al. 2005), we investigated the relationship between the designated genome-wide intra- and inter-chromosomally duplicated regions and the location of polycistronic miRNAs. The various polycistronic miRNAs resided not only in chromosomal regions involving segmental chromosomal duplications (large and small segments), but also in regions were duplications were not identified **(Figure 5)**. For instance, polycistronic miR169 precursors located in duplicated regions of chromosomes 8 and 9 (**Figure 5**). In this respect, it was previously reported that chromosomes 8 and 9 show extensive colinearity of marker loci arrangement indicating a possible common origin (Wang et al. 2000). Polycistronic miR395 precursors located on small duplication events in chromosomes 4 and 8, whereas polycistronic miR399 precursors located in segmental duplications in chromosomes 1 and 5 (**Figure 5**). Concerning the various polycistronic miR1861 precursors, two of them located within chromosomal regions with large genomic duplication events (chromosomes 8 and 9) whereas the other miR1861 precursors located in regions with no segmental duplication history (chromosomes 2, 4, 5, and 6) (**Figure 5)**. From this, it appears that only 2 out of the 6 polycistronic miR1861 precursors present in the rice genome originated by segmental duplication events. Thus, polycistronic miRNAs identified in this study located in both duplicated and non-duplicated regions of the reference genome for *O. sativa* cv. Nipponbare.

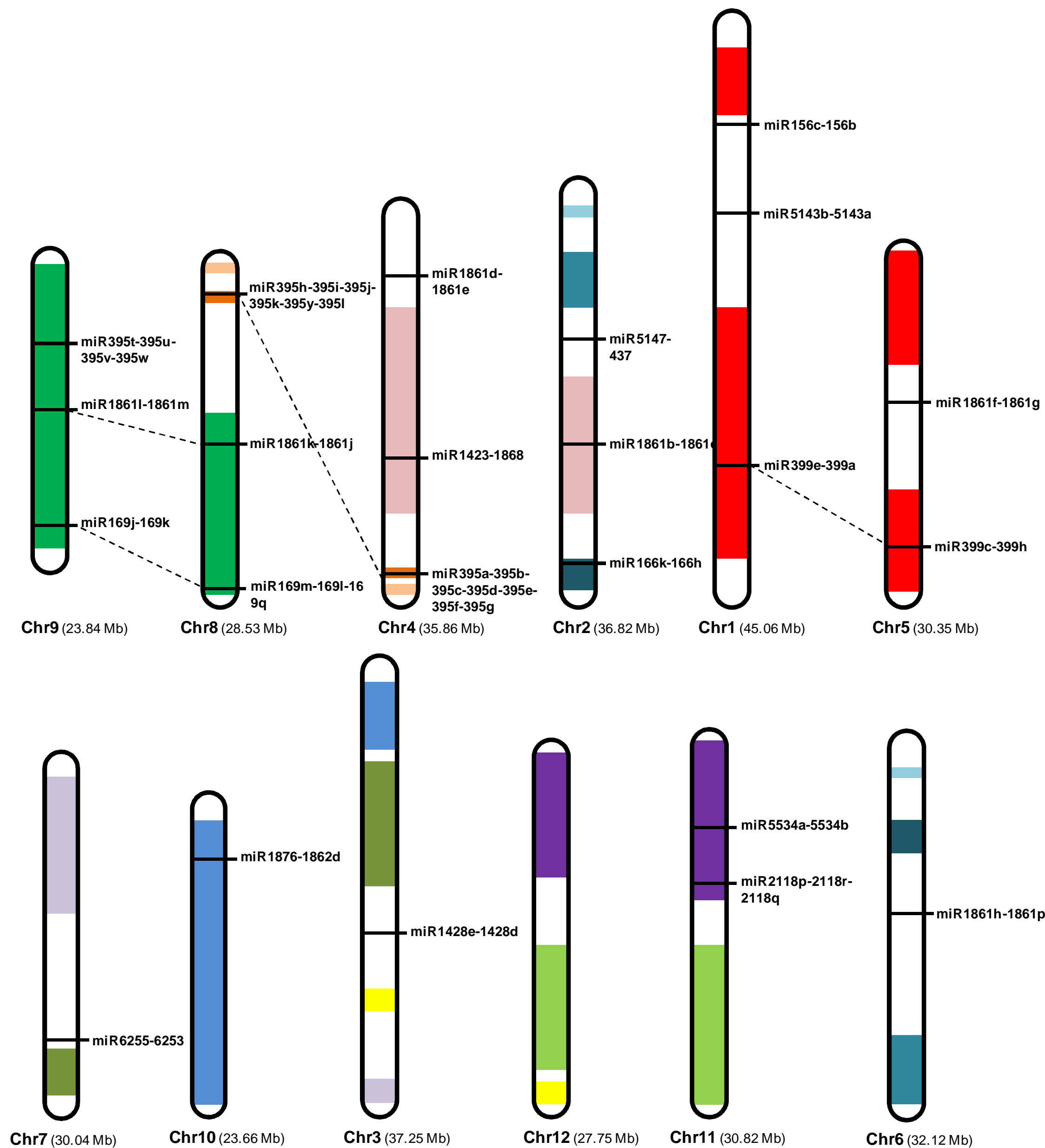


Figure 18. Chromosomal locations of polycistronic miRNAs. The relative locations of polycistronic miRNAs identified using a distance of 3kb between mature miRNAs is shown. Segmentally duplicated regions in the 12 rice chromosomes are indicated by the same color. Connecting lines indicate a correlation between duplicated regions and the presence of the indicated polycistronic precursors.

Conservation of polycistronic miRNAs in *Oryza* species

In addition to monocotyledoneous and dicotyledoneous species (see table 1), we surveyed for polycistronic miRNAs in the genome of different *Oryza* species, both cultivated and wild species. The genus *Oryza* includes species representing 10 distinct genome types, both diploid and tetraploid genomes (AA, BB, CC, BBCC, CCDD, EE, FF, GG, KKLL, and HHJJ genome types). The AA genome includes the cultivated rice and their closest relatives and ancestors.

In this work, we investigated the presence/absence of polycistronic miRNAs in *Oryza* species from different geographical regions representing the AA genome. They included: Asian species, namely *O. sativa* spp. *japonica* (cv. Nipponbare), *O. sativa* spp. *indica* (cv. 9311) and their wild relatives *O. rufipogon* and *O. nivara*; *O. glumaepatula* (cultivated in Central and South America); and *O. glaberrima* (African rice) and its wild relative *O. barthii*. Additionally, *Oryza* species representing the BB (*O. punctata*) and FF (*O. brachyantha*) genome types were included in this study. Genome data was retrieved from Ensembl Plants (version 25) (http://plants.ensembl.org/index.html).

A search in the genome of *Oryza* species revealed that only 4 out of the 23 polycistronic miRNA precursors that were identified in the genome of O. sativa japonica (cv. Nipponbare) have orthologous sequences in the genome of all the other *Oryza* species here examined (**table 3).** They were: the homologous polycistronic precursors containing miR166 species or miR395 sequences (miR166k-166h; miR395a-395b-395c-395d-395e-395f-395g and miR395h-395i-395j-395k-395y-395l), and the non-homologous miR6255-6253 polycistronic precursor. As expected, the highest sequence homology for orthologous polycistronic miRNA precursors occurred among *Oryza* species with AA genome type, their homology ranging from 78% to perfect sequence identity, depending on the precursor. While all polycistronic miRNAs were present in the genome of *O. glumaepatula* (found in America), 17 out of the 23 polycistronic miRNAs were found in the genome of the African cultivated rice *O. glaberrima* (**table 3**). Interestingly, all polycistronic miRNA loci are found in the genome of *O. barthii*, the wild relative of the African rice *O. glaberrima.*

The genome of *O. punctata,* a rice species that has a BB genome type, contains 16 orthologous polycistronic miRNAs, while only 4 orthologous polycistronic miRNAs were identified in the *O. brachyantha* genome (FF genome type). Thus, the rice species representing the BB and FF genome types appear to contain less polycistronic *MIR* genes which also have a lower similarity with those in the AA genome. This is especially notorious for *O. brachyantha* which has the smallest genome size in the *Oryza* species and is very weedy and primitive rice species.

The chromosomal location of for the various polycistronic miRNAs in the various *Oryza* species was also examined and compared to that in *O. sativa* cv. Nipponbare (chromosomal coordinates and lengths of polycistronic miRNAs in the various rice species are shown in **supplementary table S2**). When considering species that have an AA genome, the highest differences in chromosomal locations were found between the *O. nivara* genome and *O. sativa* ssp. *japonica* (8 out of the 23 polycistronic miRNAs had a different chromosomal location) (**table 3; supplementary table S2**).The chromosomal location of polycistronic miRNAs in

American species *O. glumaepatula* has a high similarity with that in the Asian rice species and their wild relatives.

Together, this study revealed that only the more advanced *Oryza* species representing AA genome types consist of most, if not all, the polycistronic *MIR* genes identified in the genome of *O. sativa* (spp. *japonica*).

Table 3. Polycistronic miRNAs in Oryza species

***O. sativa ssp Indica* (AA)**	***Oryza rufipogon* (AA)**	***Oryza nivara* (AA)**	***Oryza glumaepatula* (AA)**	***Oryza glaberrima* (AA)**	***Oryza barthii* (AA)**	***Oryza punctata* (BB)**	***Oryza brachyantha* (FF)**
98%	98%	98%	98%	-	98%	86%	-
94%	98%	98% (Chr01)	98%	98%	98%	88%	83%
98%	99%	98%	99%	97%	98%	-	-
99%	100%	98%	99%	97%	96%	-	-
99%	99%	99%	99%	90%	99%	82%	78%
99%	100%	98%	99%	97%	99%	93%	81%
100%	99%	98% (Chr01)	98%	-	98%	-	-
99%	100%	99% (Chr08)	97%	-	99%	86%	-
99%	99%	-	99%	-	99%	85%	-
-	100%	98%	98%	98%	97%	-	-
98%	99%	98% (Chr11)	99%	99%	98%	-	-
99%	100%	99%	98%	99%	94% (Chr05)	93%	-
98%	97%	97% (Chr01)	99%	99%	98%	95%	-
99%	99%	94% (Chr02)	99%	99%	96%	90%	-
99%	99%	99%	99%	98%	98%	92%	-
97%	100%	97%	98%	98%	98%	91%	-
98% (Chr04)	99%	92%	98%	99%	96% (Chr08)	95%	-
99%	99%	99%	99%	99%	99%	93%	-
99%	99%	99%	99%	99%	99%	92%	-
99%	99%	99%	99%	99%	99%	93%	-
99%	94%	99% (Chr11)	97%	-	97%	-	-
93%	90%	93%	90%	-	78%	-	-
99%	100%	99% (Chr06)	99%	97%	97%	99%	82%

Polycistronic miRNAs in *Oryza* species. *O. sativa* ssp. *japonica* corresponds to cv. Nipponbare, whereas *O. sativa* ssp. *indica* corresponds to cv. 9311. In grey, polycistronic miRNAs that are identified in the genome of the indicated *Oryza* species. For each polycistronic miRNA, its percentage of sequence homology relative to the sequence in *O. sativa* ssp. *japonica* (cv. Nipponbare) is shown. For those miRNAs that have a different chromosomal location relative to *O. sativa* cv. Nipponbare, their location is indicated. -, not present.

Discussion

Occurrence of polycistronic miRNAs is commonly found in animals. However, eventhough miRNA clusters have being identified in the genome of several plant species, the production of multiple miRNAs from the same primary transcript has been experimentally demonstrated in only a few cases (i.e. RT-PCR experiments, EST sequences).

In this study, we describe 23 polycistronic miRNAs from rice containing homologous or non-homologous miRNA species. Of them, 7 polycistronic miRNAs were experimentally validated. They were: miR166k-166h, miR1428e-1428d, miR1861b-1861c, miR1423-1868, miR1876-1862d, miR5147-437 and miR6255-6253. Here, it is worthwhile to mention that most polycistronic miRNAs so far described in plants generally contain homologous miRNAs (i.e. miR156, miR166, miR395, miR159a (Guddeti et al. 2005; Wang et al. 2007; Boualem et al. 2008; Lacombe et al. 2008; Merchan et al. 2009; Barik et al. 2014). In other studies, miR159a-159a (containing two copies of miR159a in the same precursor (Lacombe et al. 2008) was reported to be encoded in a single transcript in rice. In Arabidopsis, there are a few examples of non-homologous polycistronic miRNAs, such as miR859-774, miR397b-857, miR850-863 and miR851-771 (Merchan et al. 2009). Also in Arabidopsis, two functionally related miRNAs, miR846 andmiR842 were found to be generated by the same transcriptional unit but from alternative splicing isoforms (Jia and Rock 2013).

By analysing datasets of deep sequencing, we demonstrated that mature miRNAs contained in the rice polycistronic miRNA precursors miR166k-166h, miR1423-1868 and miR1876-1862d are co-expressed in a particular tissue and condition (leaves and roots, non-treated and elicitor-treated tissues) further supporting that these precursors represent *bona fide* polycistronic miRNAs. Concerning miR5147-437, a differential accumulation of each one miRNA species derived from this precursor is observed in leaves of rice plants. Different scenarios can be considered to explain a differential accumulation of miRNAs produced from a common precursor. Firstly, the structure of primary transcripts (pre-miRNAs) for polycistronic precursors might establish a differential processing of individual mature miRNAs through differential interaction with specific components of the miRNA biogenesis machinery. Alternatively, a different turnover rate of mature miRNAs derived from a given polycistron in a given tissue or condition might impact the relative abundance of individual mature miRNAs. Very little is known, however, on the post-transcriptional regulation of miRNAs encoded in polycistrons.

Examination of degradome sequencing data sets for rice tissues, we identified the target genes for mature miRNAs encoded in polycistrons. In this respect, it is well known that most of the miRNAs that were discovered in early reports are highly conserved throughout the plant kingdom and target transcription factors that control diverse developmental processes. Along with this, several homologous polycistronic precursors containing conserved miRNAs were found to cleave transcription factor genes involved in developmental processes and hormone signaling, such as leaf morphogenesis (miR156/OsSPL, miR166/OsHB) or flowering (miR169/Nuclear transcription Factor Y subunit).

We also identified miRNA precursors containing two or more copies of either miR395 or miR399 for which a role in the adaptive response to nutrient stress conditions is well demonstrated. Their targets, ATP sulfurylases (targeted by miR395) and PHO2 (targeted by miR399) are known to be involved in maintenance of sulfate and phosphate homeostasis, respectively (Sunkar and Zhu 2004; Guddeti et al. 2005; Kawashima et al. 2009; Hackenberg et al. 2013; Paul et al. 2015). These mineral nutrients are essential for plant growth, development, and reproduction. Accordingly, the acquisition and distribution of nutrient elements must then be tightly coordinated in plants, which might well explain thatmiR395 andmiR399 occur as large gene families in many dicots and monocots. From results here presented, it appears that *MIR399* genes can be transcribed as individual transcriptional units as well as simultaneously transcribed as polycistrons. On the other hand, a MYB transcription factor that is targeted by miR6253 in the non-homologous miR6255-6253 polycistronic miRNA has been shown to be regulated under potassium deprivation in rice (Shankar et al. 2013).

In addition to miRNAs targeting genes involved in nutrient stress, there were several polycistronic miRNAs that yield individual miRNA components controlling genes involved in abiotic stress or oxidative stress (oxidative stress is a component of most abiotic and biotic stress stimuli). Collectively, results here presented indicate that miRNAs encoded by polycistronic miRNAs regulate the expression of genes that have diverse functions during development and adaptation to environmental stress. Since co-transcription is often used to imply a functional relationship of miRNAs in polycistrons, there is the possibility that these miRNAs have evolved to fine tune gene expression in a particular pathway correlated processes giving rice to regulatory networks in the control of physiological processes. Then, it will be of interest to investigate potential functional interactions between individual miRNAs encoded by a common precursor (and their corresponding target genes).

Of particular interest are non-homologous polycistronic miRNAs. In this study, 4 non-homologous polycistronic miRNAs were identified and experimentally validated. They produce mature miRNAs that are identified only in rice (miRBase release 21) but for some of them the

target gene has not been either identified (e.g. miR1868 in miR1423-1868) or functionally characterized. A better knowledge and functional characterization of target genes for rice-specific miRNAs is then needed to infer the functional significance of the organization of these miRNAs as polycistrons.

Similar to the evolution of protein gene families, miRNAs might evolve by a process of gene duplication, including segmental duplication and tandem duplication, followed by dispersal and diversification (Maher et al. 2006; Nozawa et al. 2012). Presumably, duplication mechanisms might also account for the expansion and diversification of plant polycistronic miRNAs (Maher et al. 2006; Nozawa et al. 2012). When analysing the distribution of polycistronic miRNAs in the rice genome, we found duplicated paralogs of certain polycistronic miRNAs, such as miR1861 polycistrons, that located in both duplicated and non-duplicated chromosomal regions. However, a majority of the rice polycistronic miRNAs here identified reside in non-duplicated blocks of genomic sequences, suggesting that the contribution of genomic segmental duplication events has not been important for the expansion and diversification of these polycistronic *MIR* genes in rice. It is tentative to hypothesize that the repertoire of rice polycistronic miRNAs might have changed dynamically during evolution, perhaps in a lineage-specific manner. The possibility that evolutionarily unrelated miRNAs scattered around the genome became clustered together during evolution, i.e. by the novo formation of new miRNA hairpins in existing miRNA transcripts, to generate a polycistronic *MIR* gene should be considered. This mode of evolution has been suggested to explain the existence of clusters of microRNAs (Allen et al. 2004; Felippes et al. 2008). A different evolutionary history might have occurred among paralogs of polycistronic miRNAs in rice.

As for monocistronic miRNAs, the increase of gene copy number of polycistronic miRNAs might cause a dosage effect in the accumulation of individual miRNAs produced by these polycistrons. Since polycistronic transcription eliminates the need for individual promoter elements, polycistrons could serve to reduce the gene copy number for individual miRNAs in a particular genome with no reduction in the level of expression. Duplication events might also provide the opportunity for a polycistronic miRNA to acquire a different promoter which would then provide opportunities to generate novel spatial and/or temporal expression patterns for these miRNAs.

To further explore the evolutionary history of the rice polycistronic miRNAs identified in this work, we assessed the presence/absence of these miRNA precursors, and their chromosomal location, in the genome of different rice species. For most polycistronic miRNAs, a pattern of conservation in length, sequence and chromosomal location was found in rice species that have an AA genome (e.g. *O. sativa* ssp. *japonica*,

O. sativa ssp. *indica*, *O. rufipogon*), and at a lesser extend *O. nivara* (some of them, although present, had a different chromosomal location compared to *O. sativa* ssp. *japonica*). As for *O. glaberrima*, the African rice species with an AA genome, not all the polycistronic miRNAs were identified in its genome even though all of them are present in its wild ancestor *O. barthii*. Finally, only 4 polycistronic miRNAs were identified in the *O. brachyantha* genome (FF), whereas *O. punctata* had 16 out of the 23 polycistronic miRNAs found in *O. sativa* cv. Nipponbare. The low sequence similarity that is observed in *O. brachyantha* (FF genome) with rice species with an AA genome might be indicative of primitive forms of these *MIR* genes in the FF genome. The picture that emerges from this analysis is that polycistronic miRNA loci are spawned and lost frequently during evolution and/or domestication of rice. This is consistent with results reported by Fahlgren et al (2007) indicating that Arabidopsis *MIR* genes are undergoing relatively frequent birth and death, with only a subset being stabilized during evolution. However, we should be cautious before concluding that the observed differences among *Oryza* species are biologically relevant as for wild species only one accession has been sequenced. The obtention of high quality genome sequences for a higher number of *Oryza* accessions, both cultivated and wild relatives of *Oryza* species, is then required to get new insights into the evolutionary history of polycistronic *MIR* genes in rice.

To conclude, results here presented provide evidence on the occurrence of polycistronic miRNAs in rice. Finding novel plant polycistronic miRNAs provides a foundation for further investigation of the functional relevance of these classes of plant *MIR* genes, while allowing the identification of pathways which are regulated by miRNAs encoded by polycistrons. The simultaneous transcription of individual miRNAs within a polycistronic precursor represents a new level of complexity in miRNA functioning for regulation of gene expression. In particular, the polycistronic structure of non-homologous miRNA precursors expands their gene regulatory capacity, yet may implicate the existence of functional interactions among miRNA components operating in various biological contexts. The information here presented might be useful in future studies on the evolution and functionality of polycistronic *MIR* genes in rice a species of evident agronomical importance.

Materials and Methods

RNA isolation and RT-PCR analysis

Rice (*O. sativa* cv. Nipponbare) plants were grown at 28ºC±2ºC with a 16h/8h light/dark cycle. Leaves from three week-old plants were harvested and used for total RNA extraction using TriReagent (Ambion), followed by DNAse treatment (Turbo Free DNAse, Ambion).Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Nested RT-PCR was used for the obtention of polycistronic DNA sequences. Primers pairs for each polycistron are listed in **supplementary table S1**. Sequencing of DNA fragments confirmed the specific amplification of the entire precursor sequences whilst revealing that these precursors are transcribed as a single transcriptional unit that comprises the expected mature miRNAs.

Genome-wide identification of polycistronic miRNAs

Mature microRNAs were downloaded from miRBase registry (http://www.mirbase.org, version 21). To identify candidate polycistronic miRNAs, we used a stringent criterion of 3Kb as the interval between mature microRNAs. Distances between miRNAs were calculated by subtracting the start coordinate of the downstream microRNA and the end coordinate of the upstream microRNA. Secondary structure prediction was performed using RNAFold software with default parameters (Vienna package 2.1.0; http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

To investigate whether the polycistronic miRNAs arose or evolved from segmental duplication events, we examined their chromosomal location. The genomic map of duplicated blocks in the rice genome was taken from (Thiel et al. 2009).

Alignment of sequences and phylogenetic analysis

Polycistronic sequences were retrieved using miRBase annotation coordinates. Sequence alignment was performed using MEGA software (http://www.megasoftware.net/ - version 6 (Tamura et al. 2013)) with Neighbor Joining method. Phylogenetic tree was performed with the same software using neighbor-joining method (Saitou and Nai 1987) and divergence times for all branching points in the topology were calculated with the RealTime method. All the bars around nodes represent a 95% confidence intervals (Tamura et al. 2012).

Expression analysis of miRNAs encoded by polycistronic miRNA precursors

MiRNA expression data were obtained from the Gene Expression Omnibus (**GEO**) database and described by Baldrich et al., (2015). Data sets used in this study were retrieved from GEO (accession numbers GSM1626119, GSM1626120, GSM1626121, GSM1626131, GSM1626132, GSM1626133 for rice leaves and roots, respectively). Data sets used for the analysis of elicitor-responsiveness of miRNAs are deposited under accession number GSM1626122, GSM1626123, GSM1626124, GSM1626134, GSM1626135, GSM1626136(elicitor-treated leaves and elicitor-treated roots; 30 minutes of elicitor treatment). Finally, the degradome sequencing data sets were also retrieved from GEO (accession number GSM1626143 and GSM1626144).

Conservation analysis of polycistronic miRNAs in rice species

Genome sequences from *O. sativa* ssp. *japonica* (cv. Nipponbare) (AA), *O. sativa.*ssp. *indica* (AA), *O. rufipogon* (AA), *O. barthii* (AA), *O. glumaepatula* (AA), *O. nivara* (AA), *O. glaberrima* (AA), *O. punctata* (BB) and *O. brachyantha* (FF) were retrieved from Plant Ensembl (http://www.plants.ensembl.org, version 25). Orthologous polycistronic miRNA precursors were identified using a similarity criterion with BLAST software (version 27, Camacho et al., 2009) with default conditions.

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Supplementary Material

Supplementary Figure S1. Secondary structure of miRNA precursor structures containing two or more mature miRNAs from rice. Black arrows indicate the beginning of the folded sequences.**A.** Homologous miRNAs. **B.** Non-homologous miRNAs

Supplementary Figure S2. Nucleotide sequences of precursor structures containing two or more mature miRNAs from rice. **A.** Homologous miRNAs. **B.** Non-homologous miRNAs

Supplementary Figure S3. Secondary structure of mis-annotated miRNAs. The two mature sequences in each precursor, namely the miR1440, miR5512 or miR2275 precursors are annotated in miRBase as two different members of the corresponding family. Following the criteria for annotation in miRBase, we named these miRNAs as -5p and -3p. Black arrows indicate the beginning of the folded sequences.

Supplementary Figure S4. Nucleotide sequences of precursor structures of miR1440, miR5512 or miR2275. The two small RNAs mapping to these precursor structures are mis-annotated in miRBase as independent family members

Supplementary Figure S5. Sequence alignment of polycistronic miR1861 precursors. Sequences were aligned with the MEGA 6 software (http://www.megasoftware.net/). Black background indicates nucleotides that are identical. Dashes indicate no nucleotide present at that position. Bars indicate the locations of mature miRNAs (miRNA-5p and miRNA-3p for each precursor).

Supplementary table S1. Chromosomal locations of polycistronic miRNAs in *Oryza* species. The percentage of homology for each precursor in each rice species is shown in parenthesis. Their length is also indicated.

Supplementary table S2. List of primers used for nested RT-PCR analysis of polycistronic miRNAs.

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General discussion

General discussion

Small RNAs are key regulators of gene expression in a wide range of developmental processes, including organ polarity and morphogenesis, flowering, shoot and root development, and hormone signaling. There are also reports indicating that miRNAs are involved in the plant response to abiotic stress, oxidative stress and nutrient deficiency. In rice, the activity of certain miRNAs significantly controls traits of agronomic importance, such as tiller growth, early flowering, panicle and grain production. However, less is known about the role of the small RNA in plant-microbe interactions.

The main objective of this thesis then to bring some light into the role of small RNAs in the plant response to pathogen infection, focusing on miRNAs involved in the plant response to infection by fungal pathogens. These studies have been carried out Arabidopsis and rice plants; the two model systems used for functional genomic studies in dicot and monocot plant species.

In the studies here presented, we used fungal elicitors instead of inoculating the plant tissues with fungal spores. Elicitors are widely used to elicit plant defense responses while excluding the possibility of having small RNAs of fungal origin in the high-throughput sequencing datasets. Indeed, Li et al. 2013 recently reported rice miRNAs that that differentially respond to blast infection in resistant and susceptible rice varieties. However, in that study an important number of miRNAs were found to be of fungal origin (5%-25% depending on the interaction).

In this work, we initially examined small RNAs that are regulated by elicitors Arabidopsis using a traditional technique, such as microarray. Next, we used deep sequencing of small RNA libraries obtained from rice tissues (control and elicitor-treated tissues) for the global identification of rice miRNAs that are regulated by fungal elicitors. By combining small RNA sequencing and degradome sequencing we were able to identify distinct regulatory networks in which miRNA/target gene pairs operate in the rice response to fungal elicitors.

Concerning Arabidopsis miRNAs, we found that miR168 is transcriptionally activated by fungal elicitors, suggesting that miR168 contributes to the maintenance of

the appropriate levels of *AGO1*, and hence miRNA functioning, in the response of Arabidopsis plants. In other studies, AGO1 was shown to play an important role in antiviral defense and bacterial disease resistance. As AGO1 is an essential component for miRNA functioning, our data suggest that the observed elicitor-induced regulation of miR168 and subsequent down regulation of *AGO1* might play a fundamental role during the adaptive response of Arabidopsis plants to infection by fungal pathogens. The observation that pathogen-derived small RNAs are delivered into plant cells (*B. cinerea*-tomato/Arabidopsis interaction) and inhibit host immunity by binding to AGO1 further supports the relevance of AGO1 in plant immunity.

Our studies for the identification of elicitor-regulated small RNAs in Arabidopsis also allowed us to identify an elicitor-regulated hc-siRNA (named siRNA415). By using Arabidopsis mutants affected in small RNA biogenesis we demonstrated that this particular small RNA was incorrectly annotated in miRBase. Thus, it was annotated as a miRNA while our work revealed that it is an hc-siRNA). Hc-siRNAs are involved in methylation processes, and their main function is to maintain genome integrity by silencing transposable elements. Other reports suggest that hc-siRNAs can methylate protein coding genes via Methyltransferase 1 (MET1) and CMT3. The exact role of siRNA415 in the response of Arabidopsis plants to fungal infection remains to be investigated. In this respect, increasing evidence support a function of RNA-directed DNA methylation processes in mediating plant immune responses.

In this study, we have shown that treatment with fungal elicitors is accompanied by dynamic alterations in the accumulation of a diverse set of miRNAs in rice, both conserved and non conserved miRNAs, supporting an important role of miRNAs in shaping the plant transcriptome in rice. The target genes for elicitor-regulated miRNAs are known to be involved in a variety of biological processes, such as stress responses, development and hormone signaling, including auxin signaling. In rice, miRNAs involved in auxin signaling were found to be regulated by elicitors (miR393 and miR5488, targeting *TIR1*, miR5809 targeting *GH3,* and miR160, miR172, miR167 and miR5075 targeting ARF genes). Similar results were obtained in Arabidopsis where several miRNAs that regulate auxin homeostasis were identified among the set of elicitor-responsive miRNAs. They were: miR167 targeting two Auxin Response Factors (ARF) genes (*ARF6* and ARF8), miR164 and miR169 (targeting *NAC1* and subunits A

of the NF-Y transcription factor complex, respectively). Auxin is considered the growth-controlling hormone and in consequence, is one of the main actors in plant development. Additionally, auxin has been shown to be important in controlling resistance to *P. syringae* in Arabidopsis (Navarro et al., 2006). Collectively results obtained in this work together with those by other authors support that miRNA-mediated regulation of components of the auxin signaling pathway might function as important regulatory nodes of different developmental processes during adaptive responses to pathogen infection in plants. As miRNAs modulate gene expression by determining the steady-state of target transcripts (rather than on-off regulation) they provide a mechanism for fine-tuning of gene expression for the control of diverse processes. Presumably, this regulatory mechanism would lead to the expression of appropriate defense responses without compromising plant development. Furthermore, the regulation of defense and developmental processes by miRNAs in conserved between monocots and dicots.

To note, miRNAs that regulate the expression of components of small RNA biogenesis pathways are also regulated by fungal elicitors. They were miRNAs controlling components of the miRNA pathway itself, as well as the hc-siRNAs and ta-siRNAs pathways. We can then suggest the existence of self-regulatory mechanisms of the small RNA pathways, these regulatory mechanisms being controled by elicitor regulated miRNAs.

An important contribution of this work is the finding that elicitor-regulated miRNAs are regulators of CPuORF-containing genes in rice. Among them, we demonstrated a miR5819-mediated cleavage of *CPuORF3-bZIP38* transcripts encoding a bZIP transcription factor. Clearly, these findings add an additional layer of complexity in the refined regulatory system based on functioning of CPuORF-encoded short peptides.

As part of this work, we identified 9 previously uncharacterized miRNAs in rice (miR11336 to miR11344), as well as new members from known miRNA families' miR1861p, miR2120b, miR5801c and miR6245b. Furthermore, our degradome analysis identified targets for 6 of the 9 novel miRNAs reported in this work. The targets included disease resistance genes (RPP13, MLO), defensin, PPR proteins, ankyrin, and

vesicle-associated membrane protein genes. Further work is needed to elucidate the role of these new miRNAs in rice immunity.

To further understand the *MIR* gene organization in the rice genome, we searched for polycistronic miRNAs in rice. A total of 23 polycistronic miRNAs from rice containing homologous or non-homologous miRNA species were identified, and 7 of them were experimentally validated. By analysing datasets of deep sequencing, we demonstrated that mature miRNAs contained in most of the rice polycistronic miRNA precursors, but not all, are co-expressed in a particular tissue and condition. We further investigated the presence or absence of those miRNA polycistronic precursors in different rice species. A picture that emerges from this analysis is that polycistronic miRNA loci are spawned and lost frequently during evolution and/or domestication of rice.

To conclude, our results support that miRNAs and their corresponding target genes can be considered as an integral part of the plant response to fungal elicitors. Disease-resistant plants can be obtained by altering the expression of a particular miRNA (or its target gene) for which a role in disease resistance is known. Since pathogen attack is one of the primary causes of crop losses worldwide, unravelling the miRNA-mediated mechanisms underlying pathogen resistance of plants has profound significance. On the other hand, many of the elicitor-regulated miRNAs identified in this work, have also found to be regulated by other types of stress, including abiotic stresses. For example, miR395 and miR171 respond to several abiotic stresses and are also differentially expressed under biotic stress conditions. There is then the possibility that some of these miRNAs might be involved in cross-talk between biotic and abiotic stresses.

Some of the newly identified miRNAs from rice are being investigated in more detail in our group. Work is in progress and I will obtain transgenic rice lines overexpressing the precursor for a miRNA of interest. However, due to the long period of time that is needed to obtain homozygous transgenic rice lines (usually identified in

the T2 generation), these results have not been included in the thesis. Once these transgenic lines are obtained, their properties of disease resistance/susceptibility to infection by the rice blast fungus *M. oryzae* will be evaluated.

Other studies carried out during this period, and not included in the Thesis, refer to the identification and characterization of rice mutants affected in small RNA biogenesis. This work has been carried out in the framework of a collaboration established between our group and the group of Dr. Y-I Hsing (Academia Sinica, Taipei, Taiwan). Whereas a large number of mutants affected in small RNA biogenesis are available in Arabidopsis, this is not the case in rice. Only RNAi lines for dcl1, dcl3a, dcl3b, dcl4 and rdr6 have been produced by Chinese researchers. Clearly, the availably of these mutants will greatly contribute to the correct assignment of a small RNA as a miRNA. For that reason, during my PhD, I have been involved in studies to characterise different DCL, RDR and AGO mutants from the Taiwan Rice Insertional Mutant (TRIM) collection (http://trim.sinica.edu.tw/). TRIM mutant lines are T-DNA insertional mutants that have the potential to create gene knockouts or activation rice lines. At present, overexpressor lines for *dcl1a, dcl2a, dcl4* and *ago1* genes have been identified.

During my PhD work I also contributed to an on-going project in the group aiming the functional characterization of a gene encoding a Calcium-dependent Protein Kinase from rice, the *OsCPK4* gene. Previous results obtained in the group indicated that *OsCPK4* expression is induced by salt and drought stresses, and that *OsCPK4* overexpression results in up-regulation of genes involved in lipid metabolism and protection against oxidative stress. Overexpression of OsCPK4 in rice plants was found to enhance tolerance to salt and drought stress. My contribution to this work was on salt tolerance assays of *OsCPK4* overexpressor plants. Results from these studies were published in Plant Physiology (Campo S, **Baldrich P**, Messeguer J, Lalanne E, Coca M, San Segundo B. Overexpression of a Calcium-Dependent Protein Kinase Confers Salt and Drought Tolerance in Rice by Preventing Membrane Lipid Peroxidation. Plant Physiology 165(2): 688-704).

Conclusions

Conclusions

1. Treatment with fungal elicitors is accompanied by a dynamic response in the accumulation of miRNAs in both rice or Arabidopsis plants.

2. The microRNAs miR168, targeting *AGO1* transcripts, is transcriptionally regulated by *Fusarium oxysporum* elicitors in Arabidopsis plants, suggesting an additional layer of control of AGO1 homeostasis and, hence, miRNA functioning with plant response to fungal elicitors.

3. The hc-siRNA siRNA415, incorrectly annotated as miR415, is regulated by elicitor treatment in Arabidopsis, supporting a function of RNA-directed DNA methylation processes in plant immune responses.

4. In rice, elicitor-regulated miRNAs and their corresponding target genes control hormone pathways, such as auxin and ethylene pathways, as well as small RNA biogenesis pathways, namely ta-siRNA, hc-siRNA and miRNA pathways itself.

5. *CPuORF3-bZIP38* transcripts encoding a bZIP transcription factor are regulated by the elicitor-responsive miR5819. This finding illustrates the existence of a miRNAs-guided regulatory mechanism for the control of CPuORF containing genes.

6. We identified 9 novel from miRNAs from rice. They have been annotated with miRBase registry as miR11336 to miR11344. Four members of previously known families have been also identified in this work.

7. Polycistronic miRNA, containing homologous and non-homologous miRNA, were identified in the rice genome. Seven out of the 23 polycistronic miRNAs loci were experimentally validated.

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