UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

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BIOLOGÍA EVOLUTIVA

(PROYECTO)

Caracterización del dialogo molecular entre *Arabidopsis thaliana* - *Botrytis cinerea* mediado por microRNAs

TESIS

(POR ARTÍCULO CIENTÍFICO)

Identification of *Arabidopsis thaliana* small RNAs responsive to the fungal pathogen *Botrytis cinerea* at an early stage of interaction

QUE PARA OPTAR POR EL GRADO DE:

MAESTRO EN CIENCIAS BIOLÓGICAS

PRESENTA:

EMIR ALEJANDRO PADILLA PADILLA

TUTOR PRINCIPAL DE TESIS: Dr. Mario Alberto Serrano Ortega
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CIUDAD UNIVERSITARIA, CD. MX. FEBRERO, 2024

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COORDINACIÓN GENERAL DE ESTUDIOS DE POSGRADO
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M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e.

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **09 de octubre de 2023** se aprobó el siguiente jurado para el examen de grado de **MAESTRO EN CIENCIAS BIOLÓGICAS** en el campo de conocimiento de **Biología Evolutiva** del (la) alumno(a) **PADILLA PADILLA EMIR ALEJANDRO** con número de cuenta **313331057** por la modalidad de graduación de **tesis por artículo científico** titulado: **"Identification of *Arabidopsis thaliana* small RNAs responsive to the fungal pathogen *Botrytis cinerea* at an early stage of interaction",** que es producto del proyecto realizado en la maestría que lleva por título "**Caracterización del dialogo molecular entre *Arabidopsis thaliana-Botrytis cinerea* mediado por microRNAs**" ambos realizados bajo la dirección del **DR. MARIO ALBERTO SERRANO ORTEGA**, quedando integrado de la siguiente manera:

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Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 04 de diciembre de 2023

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Resumen

Botrytis cinerea (Botrytis) es un hongo necrotrófico de amplia distribución en todo el mundo que es capaz de infectar a una amplia variedad de especies de plantas, muchas de ellas importantes para el consumo humano. Es fundamental diseñar estrategias más efectivas para el control de este hongo. Se ha reportado que parte de la regulación de la interacción entre Arabidopsis y Botrytis es mediada por RNAs pequeños (sRNAs), que son moléculas de RNA de ~20 a 24 nucleótidos que se encuentran codificados en los genomas de los organismos. En Arabidopsis, se han identificado sRNAs que regulan diversos procesos biológicos, como desarrollo y respuestas a estrés, y en el caso de la interacción con Botrytis, se han reportado también algunos ejemplos de sRNAs de Arabidopsis que regulan la expresión de genes de Botrytis (regulación entre reinos).

Con el objetivo de identificar sRNAs que regulan la interacción entre Botrytis y Arabidopsis, particularmente durante las primeras etapas de la interacción y a una escala de transcriptoma completo, en esta tesis se muestra el análisis realizado a partir de los datos de secuenciación masiva de sRNAs de hojas de Arabidopsis tratadas con una solución control (mock) o con Botrytis.

Se muestra que de las diferentes clases de sRNAs que responden a la presencia de Botrytis, hc-siRNAs son la gran mayoría de los sRNA reprimidos en dicha condición con respecto a la condición control, pudiendo ser ésta una respuesta dirigida a mantener una estructura más relajada de la cromatina para responder de manera más eficiente al ataque de este hongo. De todos los sRNAs diferencialmente expresados, se encontró que miRNAs evolutivamente conservados son de los sRNAs más acumulados en presencia de Botrytis. Se muestra que algunos sRNAs conocidos por participar en otras interacciones patogénicas (con *Pseudomonas syringae*, con *Plectosphaerella cucumerina* o con *Phytophthora capsici*) están inducidos tempranamente en respuesta a Botrytis. En particular, hubo inducción de algunos sRNAs relacionados con la regulación de rutas de señalización de hormonas vegetales, con la regulación de DCL1 y AGO2 (enzimas que participan precisamente en las vías del silenciamiento mediado por sRNAs) y con regulación de la expresión génica entre reinos.

Abstract

Botrytis cinerea (Botrytis) is a widespread necrotrophic fungus capable of infecting a wide variety of plant species, many of them being important for human consumption. So, it is essential to design more effective strategies for its control. It has been reported that part of the regulation of the interaction between Arabidopsis and Botrytis is mediated by some small RNAs (sRNAs), which are RNA molecules of ~20 to 24 nucleotides that are encoded in the genomes of organisms. In Arabidopsis, several sRNAs regulate various biological processes, such as development and responses to stress, and in the case of the interaction with Botrytis, some Arabidopsis sRNAs regulate the expression of Botrytis genes (cross-kingdom regulation).

With the aim of identifying sRNAs that regulate the interaction between Botrytis and Arabidopsis, particularly during early stages of interaction and at a transcriptome-wide scale, in this thesis I show the analysis I performed on high-throughput sequencing data of sRNAs from Arabidopsis leaves treated with a control solution (mock) or with Botrytis.

I demonstrate that among the different classes of sRNAs that respond to the presence of Botrytis, hc-siRNAs are the vast majority of downregulated sRNAs in such condition with respect to the control condition, which may represent a response aimed at maintaining a more relaxed chromatin structure to respond more efficiently to the attack of this fungus. From all the differentially expressed sRNAs, I found that evolutionarily conserved miRNAs are among the most accumulated sRNAs in the presence of Botrytis. I demonstrate that some sRNAs known to participate in other pathogenic interactions (with *Pseudomonas syringae*, with *Plectosphaerella cucumerina* or with *Phytophthora capsici*) are induced early in response to Botrytis. I show there was induction of some sRNAs related to the regulation of plant hormone signaling pathways, to the regulation of DCL1 and AGO2 (enzymes that directly participate in the silencing pathways mediated by sRNAs) and to cross-kingdom regulation of gene expression.

Introducción

Botrytis cinerea (Botrytis) es un hongo capaz de infectar a una gran diversidad de especies de plantas, sus esporas pueden dispersarse por el aire y se encuentra muy extendido en todo el mundo (Williamson et al., 2007). Como ejemplo de los cultivos afectados se destacan: el tomate, la lechuga, el brócoli, la col, uvas, fresas, frambuesas, moras, diversas flores de ornato y diversas legumbres, así como también la planta modelo *Arabidopsis thaliana* (Arabidopsis) (Koch & Slusarenko, 1990; van Kan, 2006; Williamson et al., 2007). A Botrytis se le considera como un hongo necrotrófico pues obtiene nutrientes a partir de las células muertas de los tejidos de la planta hospedera. En muchas de sus plantas hospederas, provoca una pudrición que se caracteriza a nivel macroscópico por el ablandamiento de los tejidos infectados, el desarrollo de lesiones oscuras y, en estadios más avanzados de la infección, por el notorio crecimiento del micelio. Como ejemplo del daño causado por Botrytis, en la **Figura 1** se observan frutos de frambuesa infectados por este hongo.

Figura 1. *Infección por Botrytis en frambuesa.*
Adaptado de "*The Top 10 fungal pathogens in molecular plant pathology*" (p. 417), por Dean et al. (2012), *Molecular Plant Pathology*, 13(4).

Botrytis generalmente invade órganos o tejidos vegetales muertos, senescentes o maduros, penetrando de manera directa o a través de heridas o aberturas naturales (Dean et al., 2012; Elad, 1997; Williamson et al., 2007). Sin embargo, frecuentemente infecta a la planta hospedera durante etapas más tempranas del desarrollo de ésta, permaneciendo Botrytis en un estado quiescente hasta que el ambiente y la fisiología de la planta le son favorables (Dean et al., 2012; Williamson et al.,

2007). Esto, aunado a que este hongo puede tener actividad incluso a bajas temperaturas (Elad, 1997; Williamson et al., 2007), contribuye a que ocurran pérdidas postcosecha de una gran cantidad de productos aparentemente asintomáticos (Dean et al., 2012; Williamson et al., 2007). Dichas pérdidas pueden darse en cualquier punto de la cadena de distribución de los cultivos: tanto en el almacenamiento como en el transporte a los mercados, así como durante la exhibición y venta (Dean et al., 2012). Ya sea en campo abierto o en invernadero, Botrytis también puede estropear cultivos de manera masiva incluso antes de ser cosechados (pérdidas precosecha), afectando a algunos hospederos incluso en etapas de plántula (Dean et al., 2012; Elad, 1997; Williamson et al., 2007). Dado que este hongo causa grandes pérdidas en varios cultivos importantes para el consumo humano, es fundamental tener una mejor comprensión de los mecanismos que operan durante la interacción Botrytis-planta para diseñar estrategias más efectivas para su control.

Algunos de los factores que son determinantes para que Botrytis logre una infección exitosa sobre la planta hospedera son: las condiciones ambientales, el propio genotipo del hongo, la interacción con otros microorganismos y, desde luego, la respuesta de las plantas y su susceptibilidad (Elad, 1997). La infección por Botrytis en hojas de Arabidopsis es actualmente uno de los principales sistemas donde se estudian los mecanismos moleculares que regulan la interacción de este hongo con las plantas. Esta interacción se caracteriza por la aparición de una lesión primaria en el sitio de infección alrededor de 20 horas después de la inoculación (hpi) y que ocurre luego de la germinación de las esporas sobre las hojas, habiendo muerte local de las células vegetales debajo del sitio de penetración del hongo (Windram et al., 2012). Luego de un periodo quiescente, la infección continúa hacia una etapa tardía en la que la lesión se extiende, macerando el tejido vegetal (Prins et al., 2000).

En el transcurso de la infección, tanto en Botrytis como en la planta hospedera ocurren diversos cambios fisiológicos. Botrytis produce diferentes moléculas diferentes que contribuyen al proceso de infección (factores de virulencia) (Fillinger & Elad, 2016). Después de la germinación en la superficie de la planta, durante la penetración de la cutícula, los apresorios de Botrytis secretan enzimas que actúan promoviendo una explosión oxidativa en la interfase Botrytis-planta. Botrytis también produce una variedad de otras enzimas y metabolitos que degradan la pared celular de la planta o actúan como moléculas fitotóxicas. La planta por su parte también desencadena la producción de especies reactivas de oxígeno (ROS), lo que contribuye a la explosión oxidativa. Esta acumulación de ROS representa una perturbación del estado redox de

la planta, promoviendo una respuesta hipersensible (HR), caracterizada por la muerte celular programada en el sitio de infección. La muerte de las células vegetales (ya sea regulada, como en la muerte celular programada, o de una manera menos sutil, a través de la necrosis por compuestos fúngicos fitotóxicos) contribuye a la progresión de la infección (Koch & Slusarenko, 1990; van Kan, 2006; Williamson et al., 2007). Nuestro grupo de laboratorio ha reportado previamente que, tan temprano como a las 6 horas después de inocular Botrytis, ya hay una mayor acumulación de ROS en hojas de Arabidopsis de genotipo silvestre (Blanc et al., 2018).

Por otra parte, las plantas perciben patrones estructurales en moléculas pertenecientes al hongo (Patrones Moleculares Asociados a Microbios o a Patógenos, MAMPs o PAMPs por sus siglas en inglés), como la quitina. También pueden reconocer señales que resultan del daño causado a la propia planta (Patrones Moleculares Asociados a Daño, DAMPs por sus siglas en inglés), como los productos de degradación de la pared celular como los oligogalacturónidos. Este reconocimiento ocurre a través de receptores y correceptores ubicados en la membrana plasmática de las células vegetales (AbuQamar *et al.*, 2017; Zhou and Zhang, 2020). Una vez que las plantas perciben los patrones moleculares asociados a microbios y al daño, las señales se internalizan en la célula vegetal a través de complejas redes de señalización para activar las defensas, incluyendo cambios transcripcionales para promover la biosíntesis de moléculas antimicrobianas y la modulación de las respuestas de muerte celular (AbuQamar et al., 2017). Windram et al. (2012) reportaron que, en hojas desprendidas de Arabidopsis tratadas con Botrytis, la mayoría de los cambios en la expresión de genes (acumulación de mRNAs) se producen a las 24 hpi, cuando las lesiones permanecen pequeñas y localizadas. Recientemente, se ha reportado que parte de la regulación de la interacción entre Arabidopsis y Botrytis también es mediada por algunos RNAs pequeños (sRNAs) (Cai et al., 2018; Qiao et al., 2021; Wang et al., 2017; Weiberg et al., 2013).

Los sRNAs son moléculas de RNA de ~20 a 24 nucleótidos que se encuentran codificados en los genomas de los organismos. Algunos de estos sRNAs regulan la expresión de genes blanco a través de mecanismos guiados por complementariedad de secuencias, principalmente mediando la represión de estos blancos a nivel transcripcional (al regular la metilación del DNA y la modificación de las histonas) o postranscripcional (mediando el corte del transcrito blanco o la inhibición de su traducción) (Katiyar-Agarwal & Jin, 2010; Lee et al., 2010; Zhan & Meyers, 2023). En plantas, los sRNAs pueden clasificarse en diferentes categorías en función de sus procesos de biogénesis: hpRNAs (que derivan de precursores de RNA monocatenario con regiones

complementarias que al plegarse forman estructuras tipo tallo-asa), siRNAs (que derivan de precursores formados por dos cadenas complementarias de RNA), habiendo otras categorías menos caracterizadas, como los tsRNAs, en los cuales, los precursores son moléculas de tRNAs (Axtell, 2013; Morgado & Johannes, 2019; Zhan & Meyers, 2023). Los hpRNAs se conforman principalmente por los microRNAs (miRNAs), los cuales se procesan de forma precisa a partir de sus precursores y mayormente regulan a sus blancos a nivel postranscripcional; son la clase más caracterizada de sRNAs en las plantas. Dentro de los siRNAs encontramos otras subclasificaciones tales como siRNAs secundarios y hc-siRNAs. Los siRNAs secundarios son sRNAs producidos a partir de un transcrito primario que sufre un corte guiado por algún miRNA; alguno de los segmentos cortados es procesado a RNA de doble cadena, produciendo siRNAs secundarios por medio del corte de ese RNA de doble cadena en segmentos consecutivos de 21 nucleótidos a partir del sitio de corte. Los hc-siRNAs son generados principalmente a partir de transposones, regiones repetitivas y heterocromáticas en el genoma de las plantas y comúnmente silencian transposones a nivel transcripcional, regulando la estabilidad del genoma (Axtell, 2013; Morgado & Johannes, 2019; Zhan & Meyers, 2023) (**Figura *2***).

En Arabidopsis, se han identificado sRNAs que regulan diversos procesos biológicos, como desarrollo y respuestas a estrés (Yu et al., 2019). Hay algunos reportes que muestran que sRNAs de plantas responden a Botrytis. Jin & Wu (2015) y Liang et al. (2018) reportaron miRNAs expresados diferencialmente en presencia de Botrytis en hojas de fresa y tomate, respectivamente, identificando algunos miRNAs que potencialmente regulan positivamente la respuesta defensiva contra este hongo. Por otra parte, Weiberg et al. (2013) y Wang et al. (2017) reportaron que sRNAs también forman parte de los factores de virulencia producidos por Botrytis al infectar a Arabidopsis, los cuales pueden considerarse efectores ya que contribuyen en la inhibición de las respuestas defensivas de la planta. Estos sRNAs son secretados por Botrytis por medio de vesículas extracelulares que pueden ser incorporadas por las células de Arabidopsis por medio de endocitosis (He et al., 2023). He et al. (2023) reportaron que estas vesículas extracelulares de Botrytis ya están presentes en el sitio de la infección en hojas de Arabidopsis a las 10 hpi. Por otro lado, sRNAs de Arabidopsis también forman parte de los mecanismos de defensa utilizados por esta planta contra Botrytis (Cai et al., 2018). Cai et al. (2018) describieron que sRNAs de Arabidopsis también pueden mediar una represión genética entre reinos: sRNAs de diferentes clases se cargan selectivamente en vesículas extracelulares producidas por las células vegetales y se transportan a

las células de Botrytis; en particular, dos siRNAs secundarios pueden reprimir genes del hongo relacionados con tráfico vesicular, lo cual tiene un efecto negativo en su virulencia.

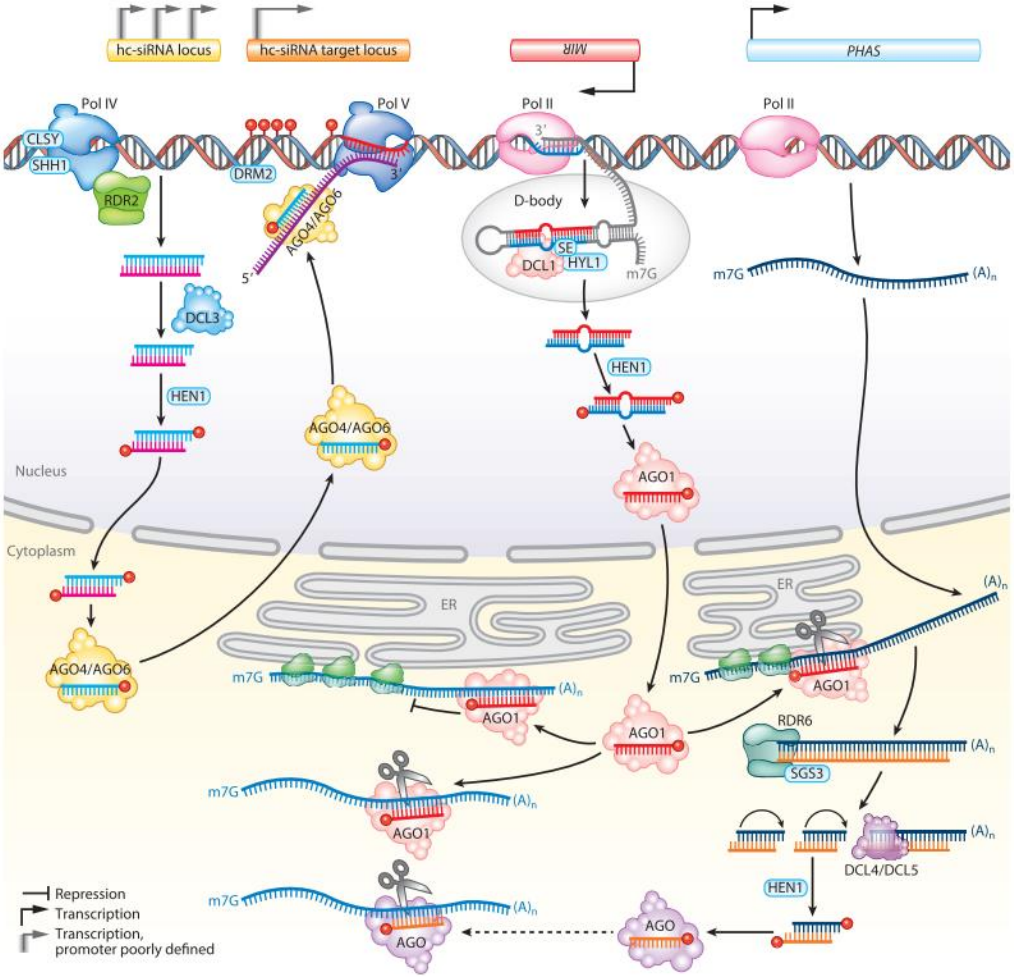

Figura 2. *Biogénesis y modos de acción de sRNAs canónicos de plantas.*
En el diagrama se muestran las principales proteínas involucradas en la biogénesis de las respectivas clases de sRNAs. Los miRNAs (centro del diagrama) y los siRNAs secundarios (a la derecha en el diagrama) pueden formar complejos con proteínas AGO y pueden mediar el corte de transcritos blancos, reprimir su traducción o desencadenar la biogénesis de otros siRNAs secundarios. Los hc-siRNAs (a la izquierda en el diagrama) forman complejos con otras proteínas AGO y principalmente median la metilación del DNA de loci transcritos por POL V. Adaptado de "*Plant Small RNAs: Their Biogenesis, Regulatory Roles, and*

Functions" (p. 3), por Zhan & Meyers (2023), *Annual Review of Plant Biology*, 74(1).

A la fecha, no hay muchos estudios a escala de transcriptoma completo sobre sRNAs en la regulación de la interacción de Botrytis con la planta hospedera. Aparte de esto, la mayoría de dichos estudios se restringen al análisis de los miRNAs de las plantas, frecuentemente dejando de lado a las otras clases de sRNAs, además de los sRNAs de Botrytis. El objetivo de este trabajo fue identificar RNAs pequeños (sRNAs) con un papel regulador en la interacción entre la planta modelo *Arabidopsis thaliana* y el hongo fitopatógeno *Botrytis cinerea*.

A continuación, se muestra el manuscrito que fue enviado a la revista Frontiers in Genetics y que versa sobre el tema de esta tesis. Posteriormente, se incluye una sección de Discusión y Conclusiones sobre este trabajo.

Artículo

frontiers

Identification of Arabidopsis thaliana small RNAs responsive to the fungal pathogen Botrytis cinerea at an early stage of interaction

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Scope Statement

Small RNAs (sRNAs), have been described as key regulators of plant development, growth, stress responses and can mediated cross-kingdom communication between plants and the necrotrophic fungus Botrytis cinerea. Even thought, the importance of this communication, to our knowledge, there are currently no transcriptome-wide studies on the regulation mediated by sRNAs during the early interaction of B. cinerea with a host plant. Moreover, most of the previous reports are limited to the analysis of specific plant miRNAs, often neglecting the other classes of sRNAs. We performed a transcriptome-wide sRNA expression analysis, on the early interaction between Arabidopsis thaliana and this pathogen. We found that mostly conserved miRNAs were the sRNAs that were upregulated in A. thaliana in the presence of B. cinerea. We performed a computational search for putative targets for the differentially expressed sRNAs between treatments. By integrating differentially expressed gene data from the same experimental conditions, we looked not only for contrasting expression profiles between treatments but also for contrasting expression profiles between sRNAs and their putative targets. The identification of sRNA-mediated regulatory circuits could help to develop sustainable strategies to mitigate fungal infection, which, ideally, could be applied not only in A. thaliana but also in other plants.

Conflict of interest statement

The authors declare a potential conflict of interest and state it below

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Keywords

A. thaliana, small RNAs, MicroRNAs, small interfering RNAs, Secondary siRNAs, mRNA target, B. cinerea infection

Abstract

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In plants, small RNAs (sRNAs), mainly microRNAs (miRNAs) and small interfering RNAs (siRNAs), have been described as key regulators of plant development, growth, and abiotic and biotic responses. Here, we performed a transcriptome-wide small RNA expression analysis, to our knowledge for the first time, on the early interaction between the necrotrophic fungus Botrytis cinerea and the model plant Arabidopsis thaliana. We found that mostly conserved miRNAs were the sRNAs that expressed the most in A. thaliana in the presence of B. cinerea. The upregulation of miRNAs miR167, miR159 and miR319 was of particular interest because these, together with their target transcripts, might be involved in the fine regulation of the plant hormone signaling pathways. Here, we also described that miR173, which triggers the production of secondary siRNAs from TAS1 and TAS2 loci, as well as TAS1 and TAS2-derived secondary siRNAs are upregulated in response to B. cinerea. Interestingly, transcript level regulation of sRNA-guided silencing machinery enzymes and of a subset of sRNA targeted genes from the PPR gene superfamily may be important for an appropriate immune response to B. cinerea at an early stage of infection. We consider that information generated in this work could be the basis for a better understanding of the regulation mediated by sRNAs during B. cinerea-plant interaction that might help developing more effective strategies for its control.

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Identification of *Arabidopsis thaliana* small RNAs responsive to the fungal pathogen *Botrytis cinerea* at an early stage of interaction

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ABSTRACT

In plants, small RNAs (sRNAs), mainly microRNAs (miRNAs) and small interfering RNAs (siRNAs), have been described as key regulators of plant development, growth, and abiotic and biotic responses. Here, we performed a transcriptome-wide small RNA expression analysis, to our knowledge for the first time, on the early interaction between the necrotrophic fungus *Botrytis cinerea* and the model plant *Arabidopsis thaliana*. We found that mostly conserved miRNAs were the sRNAs that expressed the most in *A. thaliana* in the presence of *B. cinerea.* The upregulation of miRNAs miR167, miR159 and miR319 was of particular interest because these, together with their target transcripts, might be involved in the fine regulation of the plant hormone signaling pathways. Here, we also described that miR173, which triggers the production of secondary siRNAs from TAS1 and TAS2 loci, as well as TAS1 and TAS2-derived secondary siRNAs are upregulated in response to *B. cinerea*. Interestingly, transcript level regulation of sRNA-guided silencing machinery enzymes and of a subset of sRNA targeted genes from the PPR gene superfamily may be important for an appropriate immune response to *B. cinerea* at an early stage of infection. We consider that information generated in this work could be the basis for a better understanding of the regulation mediated by sRNAs during *B. cinerea*-plant interaction that might help developing more effective strategies for its control.

INTRODUCTION

Botrytis cinerea is a widespread necrotrophic fungus with a broad host range that can infect more than 1,400 plant species, mainly angiosperms (Fillinger and Elad, 2016). Many of the affected plants are important crops for human consumption, highlighting the importance of mitigating the numerous losses caused by this pathogen worldwide each year. During infection, several physiological changes occur in both *B. cinerea* and the host plant. In many of its host plants, *B. cinerea* causes rot which is characterized at the macroscopic level by the softening of infected tissues, the development of dark lesions and, in more advanced stages of infection, the notorious growth of the mycelium (Williamson *et al.*, 2007). Two main phases of the infection have been described: the primary lesion, an early stage that follows conidial attachment and germination on the plant surface, characterized by the local death of plant cells below the fungal penetration site, and, following a quiescent period, the later stage where the lesion expands and the plant tissue is macerated (Prins *et al.*, 2000). Several factors are critical for *B. cinerea* to achieve a successful infection on the host plant: environmental conditions, the genotype of the fungus itself, interaction with other microorganisms and, the response of the plant and its susceptibility (Elad, 1997). *B. cinerea* infection on leaves of the model plant *Arabidopsis thaliana* is currently one of the main systems in which the molecular mechanisms regulating the interaction of this fungus with plants are being studied.

B. cinerea produces a number of different molecules (virulence factors) that contribute to the infection process (Fillinger and Elad, 2016). After conidial attachment and germination on the plant surface, during cuticle penetration, enzymes are secreted from *B. cinerea* appressoria whose activities promote an oxidative burst in the plant-fungal interphase. *B. cinerea* also produces a variety of other enzymes and metabolites that degrade the plant cell wall or act as phytotoxic molecules. The plant then also triggers the production of reactive oxygen species (ROS), contributing to the oxidative burst. This accumulation of ROS represents a perturbation of the redox status of the plant, promoting a hypersensitive response (HR), characterized by programmed cell death at the infection site. Plant cell death, either regulated, as in programmed cell death, or in a more unsubtle manner, through necrosis by the phytotoxic fungal compounds, contributes to the progression of infection (Koch and Slusarenko, 1990; van Kan, 2006; Williamson *et al.*, 2007).

For their part, plants can perceive patterns in molecules belonging to the fungus (such as chitin) or resulting from the damage caused to the plant (such as cell wall degradation products like oligogalacturonides) through receptors and co-receptors located in the plant cell plasma membrane (AbuQamar *et al.*, 2017; Zhou and Zhang, 2020). Once microbial and damage associated molecular patterns are perceived, the signals are internalized into the plant cell through complex signaling networks to activate defenses, including transcriptional changes to promote the biosynthesis of antimicrobial molecules and modulation of cell death responses (AbuQamar e*t al.*, 2017). Wang *et al.* (2017) and Weiberg *et al.* (2013) reported that small RNAs are also part of the virulence factors produced by *B. cinerea* when infecting *A. thaliana*, which can be considered as effectors as they can inhibit the host immune system. Small RNAs (sRNAs) are single-stranded RNAs of approximately 20 to 24 nucleotides encoded in the genomes of eukaryotic organisms which can repress gene expression, at the transcriptional or post-transcriptional level, through mechanisms guided by sequence complementarity between the sRNAs and target genes (Katiyar-Agarwal and Jin, 2010; Lee *et al.*, 2010; Zhan and Meyers, 2023).

Wang *et al.* (2017) and Weiberg *et al.* (2013) reported that some *B. cinerea* sRNAs (siR3.1, siR3.2, siR5 and siR37) can mediate cross-kingdom repression of plant immunity genes MPK1, MPK2, PRXIIF, WAK, ATG5, WRKY7, FEI2 and PMR6 by sequestration of the plant silencing protein

AGO1. *B. cinerea* secretes extracellular vesicles loaded with fungal sRNAs which can be taken up by *A. thaliana* cells by endocytosis (He *et al.*, 2023). On the other hand, plant sRNAs are also part of the defense mechanisms used by *A. thaliana* against *B. cinerea* (Cai *et al.*, 2018). In plants, sRNAs can be broadly classified into different categories according to their biogenesis mechanisms: sRNAs derived from single-stranded RNA precursors that fold into self-complementary stem-loop structures (mostly microRNAs (miRNAs)) and those derived from precursors formed by two complementary strands of RNA (siRNAs), with some other less characterized categories, such as the one in which the sRNA precursors are tRNAs (tsRNAs) (Axtell, 2013; Morgado and Johannes, 2019; Zhan and Meyers, 2023).

Cai *et al.* (2018) described that *A. thaliana* sRNAs TAS1c-siR483 and TAS2-siR453 can also mediate a cross-kingdom gene repression: these sRNAs are selectively loaded into plant extracellular vesicles and transported to *B. cinerea* cells, reducing fungal virulence by repressing vesicle-trafficking fungal genes (Cai *et al.*, 2018). There are some reports showing that plant sRNAs can also act endogenously in response to *B. cinerea*. Jin and Wu (2015) and Liang *et al.* (2018) reported differentially expressed miRNAs in the presence of *B. cinerea* in strawberry and tomato leaves, respectively, identifying some miRNAs that could positively regulate the defensive response against this fungus.

Regarding the chronology of the *A. thaliana – B. cinerea* interaction, Windram *et al.* (2012) working with *A. thaliana* detached leaves treated with *B. cinerea* reported that from the germination of the conidiophores, the fungus had an initial continuous growth of hyphae until 12 hours post inoculation (hpi), and that the first visual symptoms (primary lesion) on the leaves appear around this time. They also report that most of the changes in plant gene expression have occurred by 24 hpi, when lesions remain small and localized. He *et al.* (2023) reported the presence of *B. cinerea* extracellular vesicles (those capable of loading fungal effector sRNAs) at the infection site in *A. thaliana* leaves at 10 hpi. We have previously reported that, as early as 6 hpi with *B. cinerea*, *A. thaliana* leaves have already triggered the defense responses including ROS accumulation (Blanc *et al.*, 2018).

To our knowledge, there are currently no transcriptome-wide studies on the regulation mediated by sRNAs during the early interaction of *B. cinerea* with a host plant. Moreover, most of these studies are limited to the analysis of specific plant miRNAs, often neglecting the other classes of plant sRNAs, besides *B. cinerea* sRNAs.

With the aim of identifying small RNAs that could play a role in the early regulation of the interaction between the model plant *Arabidopsis thaliana* and the phytopathogenic fungus *Botrytis cinerea*, we analyzed the abundance of different classes of sRNAs isolated from leaf samples, comparing between control (mock treated) and *B. cinerea* treatments, using a high-throughput sequencing approach at 6 hpi. We performed a computational search for putative targets for the differentially expressed sRNAs between treatments. By integrating differentially expressed gene data from the same experimental conditions, we looked not only for contrasting expression profiles between treatments but also for contrasting expression profiles between sRNAs and their putative targets. The identification of such sRNA-mediated regulatory circuits could help to develop sustainable strategies to mitigate fungal infection, which, ideally, could be applied not only in *A. thaliana* but also in other plants.

MATERIALS AND METHODS

Growth conditions and infection of *Arabidopsis thaliana* and *Botrytis cinerea.*

We stratified *Arabidopsis thaliana* seeds (ecotype C24) for four days at 4°C and later germinated them on a substrate consisting of a mixture of peat moss Sunshine #3 mix and vermiculite (3:1) under green-house conditions at 20-22°C with a 16-h light photoperiod until plants were four weeks old. We

cultured *Botrytis cinerea* (strain B05.10) on Potato Dextrose Agar medium (PDA, 39 g/L) for approximately 20 days at room temperature. To remove hyphae, we filtered the spores and harvested them in distilled water as previously described (L'Haridon *et al.*, 2011).

We treated four-week-old adult plants with *B. cinerea* spores or with a control treatment (mock). For *B. cinerea* treatment, we adjusted the spore suspension to a concentration of 5×104 spores/mL in ¼ Potato Dextrose Broth medium (¼ PDB, 6 g/L). We incubated this concentration-adjusted suspension at room temperature for one hour before applying it to plants. For mock treatment, we used only ¼ PDB medium. We applied the treatments by spraying them over the entire surface of the plant leaves. To ensure spore germination, we kept the plants in complete darkness and high humidity conditions, covered them with plastic lids and placed them in a growth chamber at 22°C.

For each treatment, we collected approximately three fully expanded leaves per plant from 15 plants 6 hours after treatment application (6 hpi). Immediately, we froze the collected leaves in liquid nitrogen, and we stored them at -80°C until further processing. We performed the experiment twice.

RNA extraction, sequencing, and differential expression analysis.

To isolate total RNA, we ground the frozen plant tissues into powder, and we processed them according to the mirVana™ miRNA extraction kit (Invitrogen®) protocol. Subsequently, we sent the samples to Beijing Genomics Institute (BGI Americas) for the isolation of the small RNA (sRNA) fractions, library construction and sequencing for both total RNA and sRNAs.

We have previously performed the data analysis corresponding to the total RNA, such analysis can be found in Aragón *et al.* (2021). Briefly, BGI performed library construction and sequencing by DNBSEQ™ sequencing technology. We aligned sequenced reads to the reference genome of *A. thaliana* (TAIR version 10) using Bowtie2 (v2.3.5) (Langmead and Salzberg, 2012). We calculated abundances of mapped reads using the RNA-seq by expectation maximization (RSEM) method (v1.3.3) (Li and Dewey, 2011).

For sRNA analysis, sRNA library construction and sequencing were performed by BGI using the DNBSEQ™ UMI Small RNA sequencing technology. BGI also computationally filtered the raw sequences by removing adapter, contaminating and low-quality sequences. For these clean sequences, we performed an analysis to assess the quality of the data and checked the similarity between replicates in each treatment (Supplementary Figure 1). To assess whether we had recovered sequences corresponding to sRNAs in the clean reads, we analyzed the abundance distribution of the 18- to 25-nucleotide RNA sequences mapped to the *A. thaliana* genome. For these sequence alignments, we searched for perfect matches between our clean reads and the TAIR10.1 reference genome using Bowtie (v1.3.1). We observed that the 24- and 21-nucleotide sRNA species were the most abundant in both treatments (Supplementary Figure 2). This sRNA length distribution is in agreement with previous transcriptome-wide reports on *A. thaliana* and plant sRNAs (Llave *et al.*, 2002; Zhang *et al.*, 2011; Jin and Wu, 2015; Liang *et al.*, 2018; Hou *et al.*, 2019). We searched for known *A. thaliana* sRNA sequences from online resources and literature and produced a locally curated list that served as a reference for mapping the sRNA reads. Our reference list includes the mature microRNA (miRNA) sequences deposited in miRBase (version 22.1) (Kozomara e*t al.*, 2019) and sRNAanno database (Chen *et al.*, 2021), the sRNA sequences deposited in the Plant small RNA genes database (Lunardon *et al.*, 2020) and the sequences of the sRNAs delivered by *A. thaliana* to *B. cinerea* via extracellular vesicles, reported by Cai *et al.* (2018). We also included the sequences of the sRNAs delivered by *B. cinerea* to *A. thaliana* reported by Weiberg *et al.* (2013) and Wang *et al.* (2017). In total, our reference list of known sRNA sequences contains 19,027 non-redundant sRNA sequences from *A. thaliana* and 74 non-

redundant sRNA sequences from *B. cinerea*. We mapped the sRNA reads to our reference list allowing only for perfect matches and calculated their abundances using home-made scripts. We did not detect any *B. cinerea* sRNA accumulation.

We used DESeq2 (Love, Huber and Anders, 2014) with an adjusted p-value ≤ 0.05 as cutoff for the identification of differentially expressed RNAs between *B. cinerea*- and mock- treatments, both for total RNA and for sRNAs. For the differential expression analysis, we only considered those RNAs that had at least 10 read counts in both biological replicates in at least one of the two experimental conditions.

For all the expressed sRNAs in our experimental conditions, we manually curated their sequence annotations according to the class of sRNA to which they belonged. To curate the annotations, we aligned each sequence of expressed sRNA to the *A. thaliana* reference genome (TAIR version 10) using the "TAIR10 Intergenic (DNA)" and "TAIR10 Genes (+introns, +UTRs) (DNA)" databases as search sets in the TAIR-BLAST 2.9.0+ web server (https://www.arabidopsis.org/Blast/, with default parameters), to later perform an exploration in genome browser for the location and genomic context of hits with perfect matches. The curated annotations consisted of the following tags: "miRNA", "putative hpRNA", "secondary siRNA" (which included ta-siRNAs and pha-siRNAs), "hc-siRNA" (heterochromatic siRNAs), tsRNA (tRNA-derived sRNA) and "other" (for sequences that did not belong to any of the other groups due to insufficient information to assign a class tag).

Computational inference of small RNA targets and integration of RNA-seq and small RNA-seq data.

We performed computational inference of putative targets for the differentially expressed sRNAs using the psRNATarget web server (https://www.zhaolab.org/psRNATarget/) (Dai and Zhao, 2011; Dai *et al.*, 2018). We used the "Arabidopsis thaliana, transcript, removed miRNA gene, TAIR, version 10, released on 2010_12_14" cDNA sequence library available on the server to search for putative targets. We performed target inference with default parameters using the second version of the psRNATarget scoring scheme and a value of Expectation ≤ 3 as cutoff to filter the putative targets (Dai *et al.*, 2018). Using home-made scripts, we selected only the putative target sequences that appeared in the list of differentially expressed mRNAs and searched for an inverse correlation in the expression profiles between the differentially expressed sRNAs and their differentially expressed putative targets.

GO term enrichment analysis for inferred targets.

We performed a GO term enrichment analysis for the differentially expressed putative targets inferred for miRNAs, secondary siRNAs and tsRNAs; the sRNAs that most commonly exert their regulation at the post-transcriptional level. We carried out this analysis following a protocol designed for the visualization of enriched GO terms (https://bio-protocol.org/bio101/e3429). Briefly, we searched for enriched GO terms in the "Biological process" category, using the "Go Term Enrichment for Plants" tool from the online *A. thaliana* database TAIR (The Arabidopsis Information Resource). We used the *A. thaliana* gene list available on the server as reference for the algorithm. The tool utilizes PANTHER Classification System (Protein ANalysis THrough Evolutionary Relationships) (version 16.0) to run the analysis. We only considered those terms with a fold enrichment > 1 and a Benjamini-Hochberg false discovery rate value (FDR) < 0.05 calculated over Fisher exact test values. For these enriched GO terms, we performed an analysis to avoid redundancy within the terms using REVIGO (http://revigo.irb.hr/) using the *A. thaliana* GO term association frequencies reference database. We only considered those terms with a relevance similarity score (SimRel) < 0.05.

RESULTS

Evolutionary conserved miRNAs were the most accumulated *A. thaliana* sRNAs from the different classes of differentially expressed sRNAs in response to *B. cinerea* at an early stage of interaction.

To identify *A. thaliana* sRNAs that respond to *B. cinerea,* we performed a differential expression analysis of sRNAs from four-week-old plants to which we had applied mock or *B. cinerea* treatments (Table 1). In our analysis, we quantified and annotated all expressed sRNAs (regardless of whether expression was significantly different between treatments) and obtained a total of 992 non-redundant sRNA sequences expressed in both conditions (Supplementary Table 1) that belonged to different plant sRNAs classes: 605 heterochromatic siRNAs (hc-siRNAs) (~61% of all expressed sRNAs), 189 miRNAs (~19%), 23 secondary siRNAs (~2.3%), 66 tRNA-derived sRNAs (tsRNAs) (~6.6%), 8 putative hpRNAs (~0.8%) and other 101 short sequences that did not corresponded to any of the other sRNA classes (~10.2%) (Figure 1). We identified 325 differentially expressed sRNAs (185 upregulated and 140 downregulated) from *B. cinerea* treatment relative to mock treated plants (Supplementary Table 2). Interestingly, almost half the upregulated sRNAs were miRNAs, around 35% belonged to hc-siRNAs, 6% were secondary siRNAs, 3% were tsRNAs and the remaining 4% corresponded to other sequences. In contrast, 65% of the downregulated sRNAs were hc-siRNAs, 24% corresponded to other sequences, 3% were tsRNAs, only 2% corresponded to miRNAs and the remaining 6% were putative hpRNAs (Figure 2).

We checked which differentially expressed sRNAs were the most accumulated in plants treated with *B. cinerea*. Among them, the miRNAs that showed upregulated expression were miR167b/a-5p and miR167a-3p, miR166e-3p/f/b-3p/g/c/d, miR170-3p, miR171b-3p/c-3p, miR171a-3p, miR396b-5p, miR396a-5p, miR159b-3p and miR159b-5p, miR156b-3p, miR403-3p, miR172e-5p/b-5p, miR158a-3p and miR158a-5p, miR168a-3p, miR162b-3p/a-3p, miR843, miR157c-3p, miR390a-3p and miR173-5p. Although highly expressed, miR164b-5p/a showed downregulation during *B. cinerea* infection. Almost all *B. cinerea*-responsive and highly abundant miRNAs were conserved in Embryophyta (land plants), Trachaeophyta (vascular plants), Angiosperms (flowering plants) and Eudicots (flowering plants, commonly known as dicots), except miR173, miR158 and miR843 which are non-conserved miRNAs (Figure 3). Furthermore, an RNA sequence annotated as sRNA_1/6, a tsRNA annotated as sRNA_15490/18533/7401/3053, two hc-siRNAs sRNA_11003 and sRNA_12394/12395, and three secondary siRNAs: TAS2-D9(-), TAS1c-D2(+), TAS1c-siR483 were highly abundant in response to *B. cinerea* infection (Figure 3). Given the abundance and differential expression of these sRNAs, these results indicate that the early response of A. *thaliana* to *B. cinerea* infection is characterized by the upregulation and high expression of evolutionarily conserved miRNAs.

Known pathogen-responsive miRNAs and secondary siRNAs accumulated in response to *B. cinerea.*

Recent studies have previously reported plant small RNAs that could be key in response to pathogenic bacteria, fungi, and oomycetes such as *Pseudomonas syringae, Plectosphaerella cucumerina, Botrytis cinerea* and *Phytophthora capsici* (Zhang *et al.*, 2011; Soto-Suárez *et al.*, 2017; Cai *et al.*, 2018; Hou *et al.*, 2019). In response to *B. cinerea*, we found the early upregulation of some of these known pathogen-responsive sRNAs: miR319c (22 nt), miR396a-5p, miR396b-5p, miR161.2, miR167b/a, miR167c-5p and miR159b-3p, and the secondary siRNA TAS1c-siR483 (Figure 4). In particular, miR167, miR159 and miR319c regulate transcripts of genes involved in the biosynthesis and signaling pathways of the plant hormones auxin, abscisic acid and jasmonic acid in *A. thaliana* (Zhang *et al.*, 2011). Therefore, we checked whether the expression profiles of miR167, miR159, miR319c target

genes exhibited downregulation in our data from mock and *B. cinerea* treatments. The Auxin Response Factor 8 (ARF8) gene, targeted by miR167, was indeed downregulated during *B. cinerea* infection (Supplementary Figure 3). Regarding miR159 target genes, MYB65 did not show any changes in its expression profile, and we could not detect MYB101 expression. However, one isoform of MYB33 (AT5G06100.3) was downregulated in *B. cinerea* treatment at 6 hpi (Supplementary Figure 3). miR319 targets transcripts of the TCP gene family, which encodes transcription factors primarily related to development, and control of the biosynthesis of the plant hormone jasmonic acid (Palatnik *et al.*, 2003; Schommer *et al.*, 2008). In our analysis, TCP2 (AT4G18390.1 and AT4G18390.2) and TCP10 (AT2G31070.1), both target genes of miR319c, were also downregulated upon *B. cinerea* infection (Supplementary Figure 3). These results suggest that these miRNAs could be involved in the regulation of immunity by indirectly fine-tuning the plant hormones auxin, abscisic acid and jasmonic acid in *A. thaliana* during *B. cinerea* infection.

On the other hand, Cai *et al.*, (2018) described that TAS1c-siR483 and TAS2-siR453 (secondary siRNAs derived from TAS1c and TAS2 *A. thaliana* loci), are selectively loaded into plant extracellular vesicles, transported to the *B. cinerea* cells, and reduce fungal virulence by mediating cross-kingdom repression of vesicle-trafficking genes. Interestingly, we found that miR173, which triggers the production of secondary siRNAs from TAS1a, TAS1b, TAS1c and TAS2 (Allen *et al.*, 2005; Yoshikawa *et al.*, 2005), was slightly upregulated upon *B. cinerea* treatment (6 hpi) (Figure 5). We also found the upregulation of secondary siRNAs derived from TAS1c and TAS2 transcripts during *B. cinerea* treatment (Supplementary Figure 4 and 5, and Figure 5). We detected the upregulation of the TAS1c-derived secondary siRNAs TAS1c-3' D2 (+) (highly abundant), siR602, S16971, siR483 (highly abundant) and siR196, and although we did not detect expression of TAS2-siR453 in any of the treatments; we did detect the upregulation of the TAS2-derived secondary siRNAs siR710, S19300, TAS2-3' D9 (-) (highly abundant) and siR165 with *B. cinerea* treatment compared to mock (Figure 5). Taken together, these results indicate that sRNAs transported into *B. cinerea* cells via extracellular vesicles produced by *A. thaliana*, are already induced in the plant at this time point of the interaction (6 hpi).

Putative mRNA targets for the differentially expressed sRNAs.

To evaluate the processes in which the differentially expressed sRNAs are involved, we performed a computational analysis using psRNATarget to search for putative mRNA targets for these sRNAs. Using the data from our previous publication where we worked on the transcriptome characterization of *A. thaliana* in the same conditions of mock and *B. cinerea* treatment at 6 hpi (Aragón *et al.*, 2021), we searched for the differentially expressed mRNAs and found 6,247 and 5,997 upregulated and downregulated mRNAs, respectively (Supplementary Table 3 and 4 and Supplementary Figure 6). We selected only those sRNAs and their putative mRNA targets that showed the inverse correlation in their expression profiles. We found 499 cases exhibiting this type of correlation. Integrated results of differentially expressed sRNAs and anticorrelated differentially expressed mRNA inferred targets are available in Supplementary Table 5.

To identify the biological processes in which *A. thaliana* differentially expressed sRNAs and their putative mRNA targets are involved during *B. cinerea* infection, we performed a Gene Ontology (GO) term enrichment analysis. The latter included target transcripts of miRNAs, secondary siRNAs, and tsRNAs with contrasting expression patterns. In general, we found the enrichment of GO terms related to different processes such as biological regulation, cellular and metabolic processes, response to stimulus, signaling, cell communication, somatic embryogenesis, cellular response to sulfur starvation

and primary miRNA processing (Figure 6). The enriched GO terms and the respective associated genes can be consulted in Supplementary Table 6.

miRNA-mediated regulation of DCL1 and AGO2 as part of the early response to *B. cinerea*.

Surprisingly, we found the high enrichment of the GO term "primary miRNA processing (GO:0031053)" (Figure 6). One of the four target genes associated with this term was *DCL1* (AT1G01040), a gene whose protein product is an enzyme involved in the biogenesis of miRNAs, specifically in the processing of primary transcripts and the production of mature miRNAs (Morgado, 2020). *DCL1* is transcribed in a pre-mRNA transcript containing twenty exons. Full-length *DCL1* mRNA can be post-transcriptionally regulated by miR162, with the corresponding cleavage site formed by the junction of exons 12 and 13 (Xie *et al.*, 2003). In our analysis, miR162b-3p/a-3p is upregulated and is anticorrelated with the downregulation of *DCL1* transcript in *B. cinerea* treatment (Figure 7). Furthermore, the enriched GO term "response to stimulus (GO:0050896)" was associated with another sRNA-mediated gene silencing related gene: AGO2 (AT1G31280). It has been described that AGO2 is an RNAi effector gene involved in plant antiviral defense, and that its transcripts can be regulated by miR403 (Allen *et al.*, 2005; Harvey *et al.*, 2011). In our analysis, miR403-3p was upregulated and *AGO2* was downregulated by *B. cinerea* infection (Figure 7). Taken together, these results suggest that post-transcriptional regulation of *DCL1* and *AGO2* transcripts, and thus sRNA-mediated silencing pathways, could be important for *A. thaliana* to mediate the early response to *B. cinerea*.

miR161.2, miR400, TAS2-siR165 and a subset of PPR genes respond to *B. cinerea* at an early stage of the infection.

One of the genes associated with the GO term "biological regulation (GO:0065007)", AT5G55840, belongs to the Pentatricopeptide repeat (PPR) gene superfamily, which consists of more than 400 members in plants. What is currently known for some PPR proteins is that they perform their functions mainly in mitochondria or chloroplasts, binding one or several organellar transcripts, and influencing their expression by altering RNA sequence, turnover, processing, or translation (Barkan and Small, 2014). It has been reported that PPR genes are post-transcriptionally regulated by sRNAs and could be involved in the regulation of the *A. thaliana* immune response: Park *et al.* (2014) reported that miR400 regulates AT1G62720 PPR gene and knockdown mutants of this PPR gene are more susceptible to *B. cinerea.* The sRNAs miR161.1, miR161.2, miR400 and some TAS1a/b/c- and TAS2-derived secondary siRNAs (which in turn are produced after miR173-mediated cleavage of their TAS precursor transcripts) can regulate several *A. thaliana* PPR genes (Allen *et al.*, 2004; Howell *et al.*, 2007; Park *et al.*, 2014). Among the differentially expressed sRNA putative targets, we found that AT5G55840 together with AT1G62914, AT5G41170 and AT5G65560 PPR genes were predicted to be targets of miR161.2 (Supplementary Table 5). The cleavage of AT1G62914 and AT5G41170 by miR161.2 has been confirmed (Allen *et al.*, 2004; Vargas-Asencio and Perry, 2020), whereas the cleavage of AT5G65560 has not yet been validated. In our laboratory we have confirmed the cleavage-mediated regulation by miR161.2 of AT5G55840 (Ana Karen Avila-Sandoval, personal communication, June, 2023). In addition to miR161.2 cleavage, we also found that AT1G62720 and AT1G62914 gene were regulated by miR400 and TAS2-siR165, the latter also regulating AT5G41170 (Supplementary Table 5). In our *B. cinerea*-treated plants, miR161.2, miR400 and TAS2-siR165 were up-regulated (Figure 5 and Figure 8) and, although AT1G62720 was not differentially expressed between the treatments, we found that AT1G62914, AT5G41170, AT5G55840 and AT5G65560 transcripts were downregulated, compared to mock-treated plants (Figure 8). These results suggest that miR161.2, miR400 and miR173/TAS2-siR165 mediate the regulation of a subset of PPR genes during early *B. cinerea* infection in *A. thaliana.*

DISCUSSION.

With the aim of identifying *A. thaliana* early responsive small RNAs to *B. cinerea* infection, we sequenced and compared the expression levels of sRNAs from four-week-old plant leaves treated with *B. cinerea* or mock at 6 hpi. We found that different classes of sRNAs (hc-siRNAs, miRNAs, secondary siRNAs, tsRNAs and putative hpRNAs) were responsive to *B. cinerea*.

Early contribution of hc-siRNAs in the *A. thaliana* response to *B. cinerea.*

Mostly generated from transposons, repetitive and heterochromatic regions, hc-siRNAs commonly silence transposons at a transcriptional level by regulating DNA methylation and histone modification, thus contributing to genome stability (Borges and Martienssen, 2015; Yu *et al.*, 2020). A link between DNA demethylation and activation of plant immunity has been reported, which may be caused by a greater ease of expression of defense related genes due to the DNA hypomethylation and subsequent relaxation of the chromatin (Hannan Parker *et al.*, 2022). We found that hc-siRNAs represented an important proportion from the non-redundant differentially expressed sRNAs at 6 hpi, accounting for 65% of the downregulated sRNAs with *B. cinerea* treatment. Thus, an early downregulation of the hc-siRNAs in *A. thaliana* could be an important mechanism during the interaction with *B. cinerea*, allowing the plant to enhance the expression of defense related genes.

Early expression regulation of plant hormone regulatory miRNAs during *B. cinerea* infection.

Activation of plant immunity is also often correlated with the upregulation of plant hormone regulatory miRNAs, as there is a trade-off between plant growth and defense activities (Qiao *et al.*, 2021). In our results, during early *B. cinerea* infection, almost half the upregulated sRNAs were miRNAs in striking comparison to only 2% of the downregulated sRNAs. We found the upregulation of miR167, miR159 and miR319, which could be mediating an early response through auxin, abscisic acid (ABA) and jasmonic acid (JA) signaling pathways, respectively, in *A. thaliana* during *B. cinerea* infection. In agreement with the importance of these miRNAs in the biotic stress response, Zhang *et al.*, (2011) reported that *A. thaliana* inoculated with an avirulent strain of the bacterial pathogen *Pseudomonas syringae* pv. *Tomato* carrying the effector protein avrRpt2 showed an upregulation of miR159 and miR319 at 14 hpi. They reported the concomitant downregulation of the respective target genes, thus suggesting the repression of the components involved in the ABA and JA signaling pathways and the subsequent enhancement of the salicylic acid (SA)-mediated defense. Furthermore, pointing out a role of these miRNAs in the interaction with *B. cinerea*, Jin and Wu (2015) and Wu *et al.*, (2020) showed that sly-miR159 and sly-miR319 and their respective target genes are also upregulated and downregulated, respectively, in tomato during fungal infection. They also reported that *A. thaliana* overexpressing miR319c showed not only the downregulation of the miR319c target gene TCP2, but also an increased resistance to *B. cinerea* infection, pointing out TCP2 as a negative regulator of *A. thaliana* resistance to *B. cinerea* infection. Taken together, these data indicate that expression regulation of miR159, miR319 and their target genes is important for activating appropriate defenses during an early stage of interaction between *A. thaliana* and *B. cinerea*.

Known cross-kingdom *A. thaliana* miRNAs and TAS-derived secondary siRNAs as early responsive sRNAs to *B. cinerea.*

Recently, Cai *et al.*, (2018) and Zhang *et al.*, (2016) reported that plant cells export sRNAs into fungal cells by means of extracellular vesicles, mediating cross-kingdom gene silencing of fungal genes, contributing to disease resistance. Cai *et al.*, (2018) showed that the TAS1c- and TAS2-derived secondary siRNAs TAS1c-siR483 and TAS2-siR453 are selectively loaded into plant extracellular

vesicles to be transported to *B. cinerea* cells for the silencing of fungal virulence factor genes as part of the *A. thaliana* defense mechanisms. They also detected TAS1c-siR483, TAS2-siR453, IGN-siR1 (hc-sRNA) and miR166 within *B. cinerea* protoplasts purified from *B. cinerea*-infected *A. thaliana*. Zhang *et al.*, (2016) reported that cotton miRNAs miR166 and miR159 are also capable of cross-kingdom silencing of virulence-related genes, in this case, with the fungal pathogen *Verticillium dahliae*. They showed that these two plant miRNAs were present inside the fungal cells and induced upon infection, while their putative fungal target genes, Ca^{2+}-dependent cysteine protease (Clp-1) and isotrichodermin C-15 hydroxylase (HiC-15), were downregulated in fungal hyphae. In our results, we found that TAS1c-siR483, miR166e-3p/f/b-3p/a-3p/g/c/d/h/i and miR159b-3p were upregulated in response to *B. cinerea*, being part of the topmost abundantly expressed sRNAs (Figure 3). Taken together, these data suggest that the upregulation of vesicle-transported sRNAs TAS1c-siR483 and possibly miR166, and miR159 is important in early cross-kingdom gene silencing defense response.

Early expression regulation of sRNA-mediated silencing related genes by sRNAs during *B. cinerea* infection.

In this study, we explored GO term enrichment analysis for the putative target genes of the differentially expressed sRNAs. The GO term enrichment analysis revealed that target genes are involved in different biological processes. Notably, we found high enrichment for the GO term "primary miRNA processing (GO:0031053)". One of the genes associated with this term was *DCL1*, which is regulated by miR162. Zhang *et al.*, (2015) reported in rice the downregulation of OsDCL1 and the upregulation of osa-miR162a when treated with the rice blast pathogen *Magnaporthe oryzae*. They also reported that rice OsDCL1 RNAi lines constitutively expressed defense related genes and were also more resistant to virulent strains of *M. oryzae*, hypothesizing a negative role for OsDCL1 in rice immunity. Here, we report that upregulation of miR162b-3p/a-3p and downregulation of *DCL1* occurs at an early stage of *B. cinerea* infection. Rajagopalan *et al.*, (2006) reported that in addition to miR162, miR838 is another miRNA with the potential to regulate *DCL1* post-transcriptionally. They reported that this miRNA derives from a hairpin within the 14th intro of the DCL1 pre-mRNA and proposed that the presence of this intronic miRNA enables a self-regulatory mechanism that helps maintaining DCL1 homeostasis in *A. thaliana*. In our results, we found the upregulation of miR838 with *B. cinerea* treatment (Supplementary Figure 8), and we also propose other possible targets for miR838 (Supplementary Table 5) for which it could be interesting to validate the post-transcriptional regulation during early *B. cinerea* infection. Additionally, we noticed there was another sRNA biogenesis related gene associated with an enriched GO term; in this case AGO2 was associated with the "response to stimulus (GO:0050896)" term. Harvey *et al.*, (2011) have characterized AGO2 as an antiviral defense gene that responds to several plant viruses. They mentioned that AGO1 represents a first layer of RNA-mediated defense in the interactions between plants and viruses, which can be inactivated by some viruses that produce AGO1 silencing suppressors. They also mention that this therefore activates a second layer of RNA-mediated defense where AGO2 mRNA is no longer suppressed by AGO1-associated miR403. In our results, miR403-3p was upregulated and AGO2 was downregulated by *B. cinerea* infection. Taken together, these results suggest that post-transcriptional regulation of DCL1 and AGO2 transcripts, and therefore of the sRNA-mediated silencing related genes, could be important for appropriate immune responses in *A. thaliana* at an early stage of interaction with *B. cinerea*.

PPR gene targeting by miRNAs and TAS-derived secondary siRNAs as an early plant responsive mechanism to *B. cinerea*.

Associated with the enriched GO term "biological regulation (GO:0065007)", we found a PPR gene (AT5G55840) for which it has been previously validated to be regulated by miR161.2 (Ana Karen Ávila Sandoval, personal communication, June, 2023). Recently, Hou *et al.* (2019) found that miR161, which triggers the production of secondary siRNAs derived from a subset of PPR gene transcripts, contributes to *A. thaliana* defense against the oomycete pathogen *Phytophthora capsici*. They reported that miR161 and the PPR-derived secondary siRNAs, PPR-siRNA-1 and PPR-siRNA-2 were upregulated upon challenge with *P. capsici*. They also reported that *A. thaliana* lines overexpressing *MIR*161 showed enhanced resistance to *P. capsici* while *MIR*161 knock-out lines were hypersusceptible. They also showed that *A. thaliana* mutant lines of *RDR6* and *SGS3* (enzymes involved in the secondary siRNA production) were also hypersusceptible to the pathogen, suggesting a role for the secondary siRNA pathway in plant defense during *P. capsici* infection. They reported that these PPR-derived secondary siRNAs can be found in extracellular vesicles and likely silence target genes in *P. capsici* during natural infection. In this sense, future work will allow us to better understand the regulation of miR161.2, miR400 and TAS2-siR165 on PPR genes (such as AT1G62914, AT5G41170, AT5G55840 and AT5G65560), which possibly participate in the production of PPR-derived secondary siRNAs that silence target genes in *B. cinerea*.

CONCLUSION.

During early *B. cinerea* infection (6 hpi), *A. thaliana* induces changes in the accumulation of different types of sRNAs. Among these sRNAs, miRNAs were mainly upregulated. Some of the corresponding mRNA targets are involved in various biological processes such as hormone response and production of secondary siRNAs derived from *TAS1/2* genes and a subset of PPR genes. Some of the differentially expressed sRNAs are known to be transported in extracellular vesicles into fungal cells likely silencing fungal virulence factors. *A. thaliana* also post-transcriptionally regulates the levels of *DCL1* and *AGO2*, key components of sRNA-mediated silencing pathways, which could be important for an appropriate immune response of *A. thaliana* against *B. cinerea* infection.

Data Availability Statement.

The sRNA sequencing files can be found in NCBI BioProject: PRJNA1010334; https://dataview.ncbi.nlm.nih.gov/object/PRJNA1010334?reviewer=u3g871cjhvnllr621l98alr7rk.

Conflict of Interest.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions.

EAPP, CDlR, DF, and MS set up the experimental design. WA, AKÁS supervised plant growth, *B. cinerea* infection, tissue collection and RNA extractions. EAPP carried out the bioinformatic analysis and data processing. EAPP, CDlR, AEDA, MAV, DF, and MS contributed to the data interpretation, drafting, and critical revisions of the work.

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Table 1. Number of sequenced reads in each library, Mock and *B. cinerea* treatment

Read lengths	**Mock R1**	**Mock R2**	***Botrytis* R1**	***Botrytis* R2**
Raw (50 nts)	13,380,937	20,170,429	24,135,389	22,139,831
Clean (18-45 nts)	10,663,953	14,612,605	14,846,111	14,776,668

FIGURES LEGENDS.

Figure 1. Abundances of the different classes of non-redundant plant sRNAs. We found a total of 992 non-redundant expressed sRNAs that were present in our four libraries. The classes identified were heterochromatic siRNAs (hc-siRNAs), miRNAs, secondary siRNAs, putative hpRNAs and tRNA-derived sRNAs (tsRNAs) among the expressed sRNAs and other short sequences that did not correspond to any of the other classes. Numbers represent counts of non-redundant plant sRNAs.

Figure 2. Differentially expressed *A. thaliana* sRNAs. The left graph shows differentially expressed sRNAs between treatments *B. cinerea* compared to mock. Blue and grey dots are sRNAs with and without statistically significant differences in expression, respectively, (p-value ≤ 0.05). The numbers at the corners indicate upregulated (above) or downregulated (bottom) sRNAs. The right graphs show the sRNAs class abundances of upregulated (above) and downregulated (bottom) sRNAs. Numbers in pie charts represent counts of non-redundant plant sRNAs.

Figure 3. Evolutionary conserved miRNAs and some known *B. cinerea*-responsive *TAS* loci-derived secondary siRNAs represented the majority of the differentially expressed *A. thaliana* sRNAs among the most accumulated in response to *B. cinerea* at 6 hpi. Some of these miRNAs are conserved in the Embryophyta (green), Trachaeophyta (gray), Angiosperms (blue) and Eudicots (dark green) plant lineages. Histograms show all differentially expressed sRNA (adjusted p-value ≤ 0.05) which had at least an average of 1000 normalized counts in the *B. cinerea* condition. Normalized counts were obtained using the DESeq2 algorithm. Histograms indicate the mean of the normalized expression values of two biological replicates, the error bars represent the standard deviation.

Figure 4. Differentially expressed known pathogen responsive sRNAs at 6 hpi with *B. cinerea* treatment. Bars indicate the fold change of the expression levels of the differential expressed sRNAs (adjusted p-value ≤ 0.05) miR319c (22 nt), miR396a-5p, miR396b-5p, TAS1c-siR483, miR161.2, miR167b/a, miR167c-5p and miR159b-3p in *B. cinerea* treatment compared to mock, the error bars represent standard error.

Figure 5. miR173-5p as well as some known *A. thaliana* extracellular vesicle-loaded TAS-derived secondary siRNAs are upregulated during early *B. cinerea* infection. TAS derived-secondary siRNAs that are loaded in *A. thaliana* extracellular vesicles are indicated with different colors in the respective dsRNA diagrams. The plot shows the expression levels of miR173-5p and TAS1c and TAS2-derived secondary siRNAs between mock and *B. cinerea* treatments. Normalized counts of expression values were obtained using the DESeq2 algorithm. Error bars represent standard deviation of two biological replicates. Differential expression between mock and *B. cinerea* treatment is indicated by asterisks (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***), or no significant (N.S.)).

Figure 6. Biological processes in which the putative mRNA targets for sRNAs (miRNAs, secondary siRNAs, and tsRNAs) are involved. The putative mRNA targets used in the GO term enrichment analysis have contrasting patterns of expression with their corresponding regulatory sRNAs. The plot shows the enriched GO terms for biological process (FDR < 0.05), the color of the circles indicates the FDR value of each enriched term and the number of genes associated with each term is indicated under each circle.

Figure 7. small RNA-mediated gene silencing genes and their regulatory sRNAs are regulated during early *B. cinerea* infection. Expression profiles of miR162b-3p/a-3p and DCL1 (left) and expression profiles of miR403-3p and AGO2 (right) between mock and *B. cinerea* treatments. Normalized counts were obtained using DESeq2. Error bars represent standard deviation of two technical replicates. Differential expression between mock and *B. cinerea* treatment is indicated by asterisks (p-value < 0.05 (*) and < 0.001 (***)).

Figure 8. Regulation of the expression of miR161.2, miR400, TAS2-siR165 and their respective PPR gene targets during early *B. cinerea* infection. Normalized counts of expression values were obtained using DESeq2. Error bars represent standard deviation of two technical replicates. Differential expression between mock and *B. cinerea* treatment is indicated by asterisks (p-value < 0.05 (*), < 0.01 (**) and < 0.001 (***), or no significant (N.S.)). Filled and empty arrows connect miRNA with confirmed and putative PPR target genes, respectively.

Figure 1. *Abundances of the different classes of non-redundant plant sRNAs.*

Figure 1.TIFF

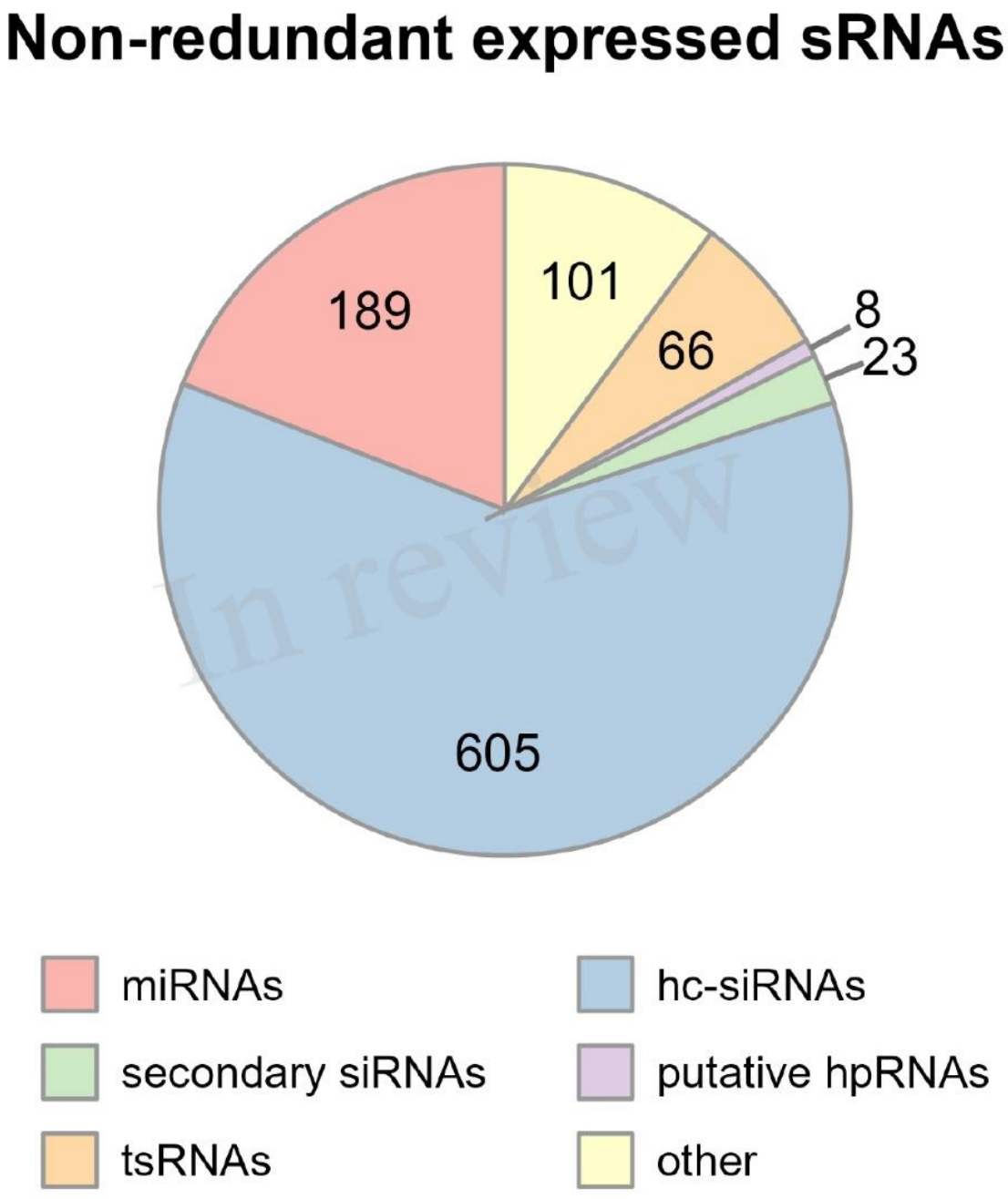

Figure 2. *Differentially expressed* A. thaliana *sRNAs.*

Figure 2.TIFF

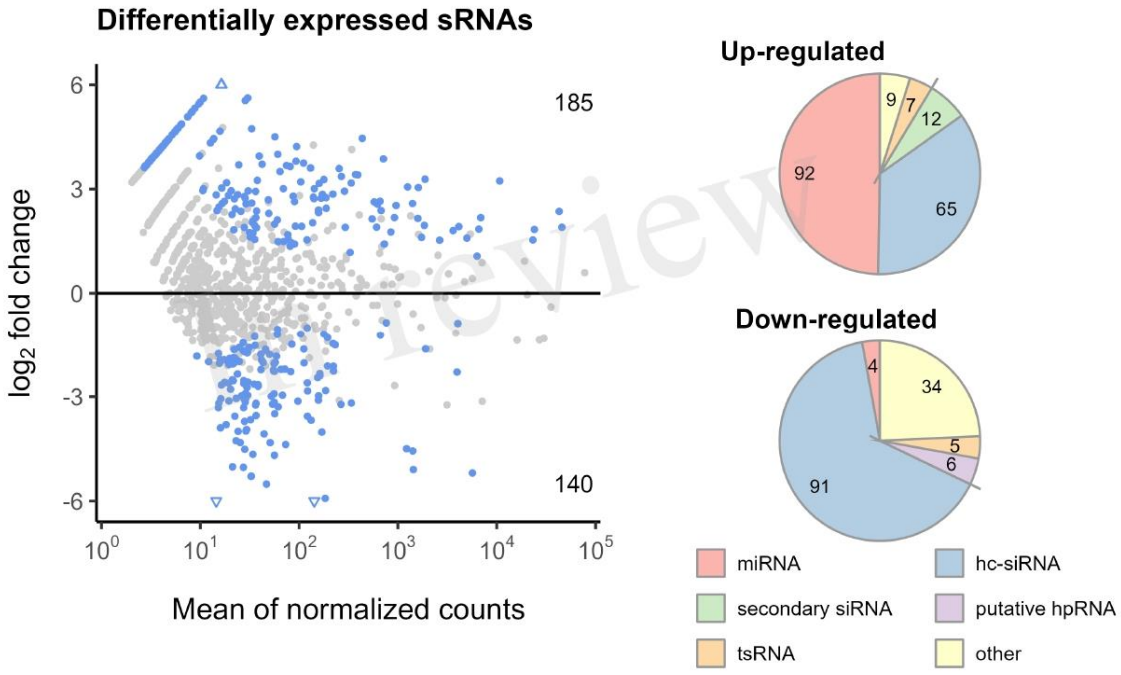

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Figure 3.TIFF

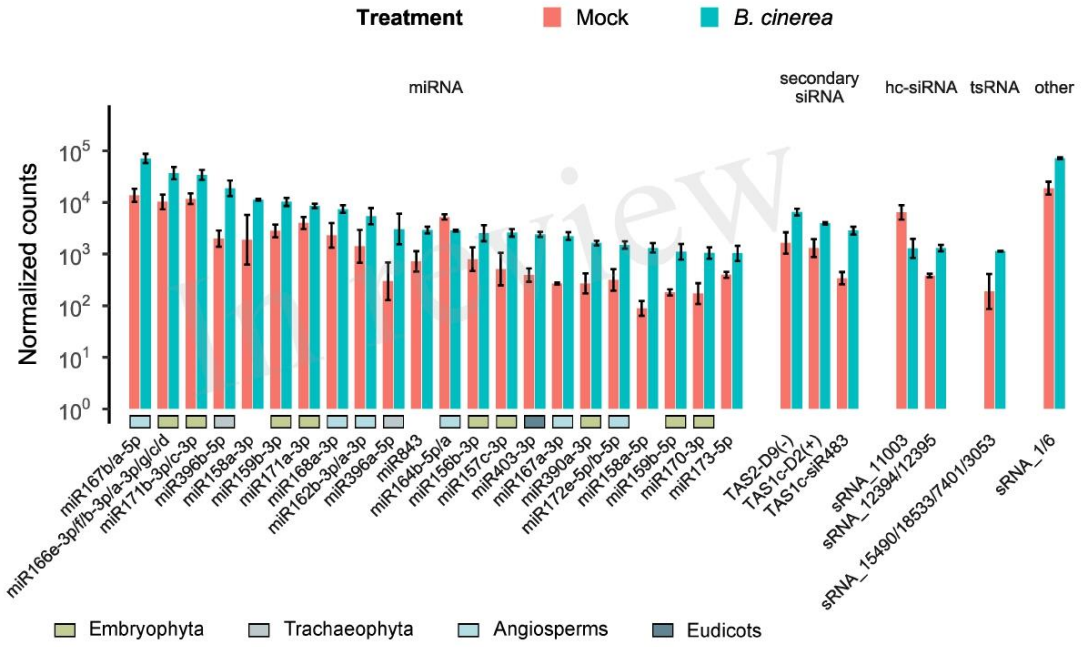


Figure 4. *Differentially expressed known pathogen responsive sRNAs at 6 hpi with* B. cinerea *treatment.*

Figure 4.TIFF

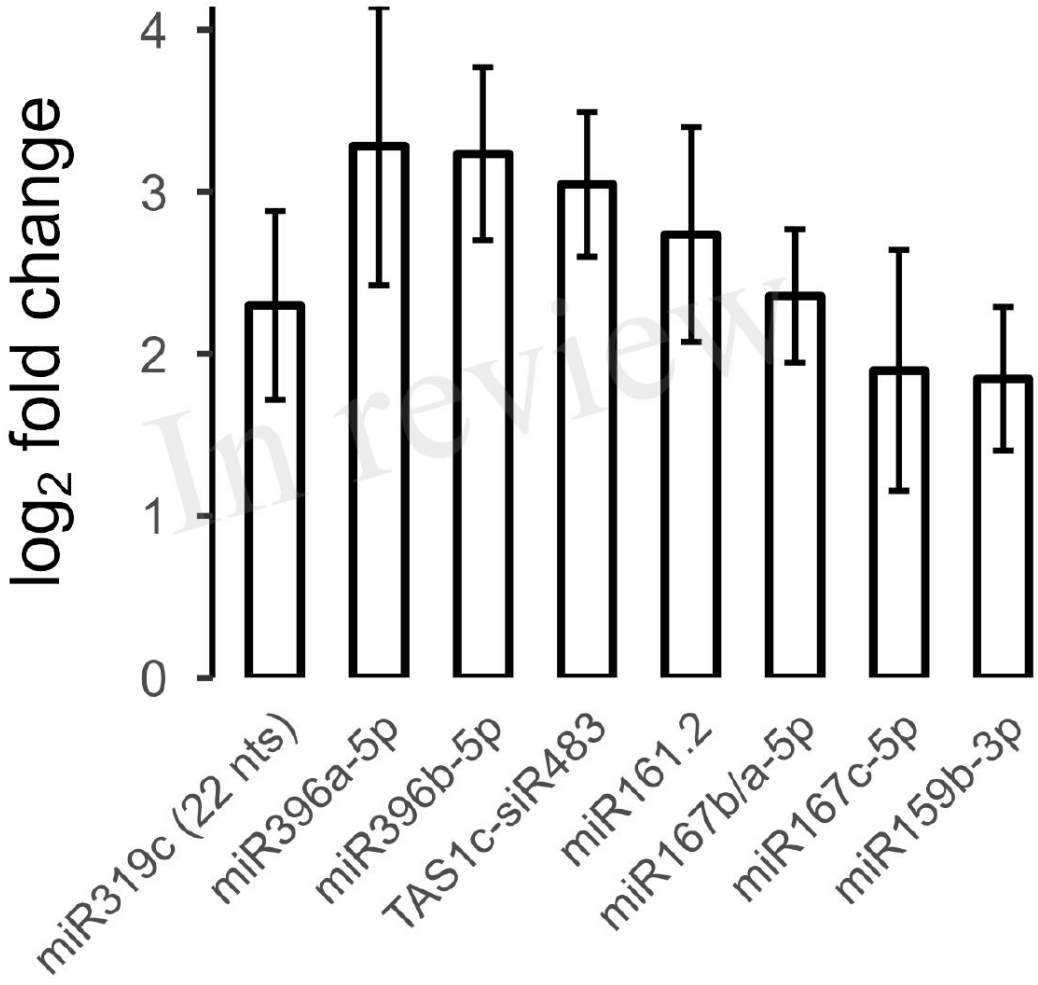


Figure 5. *miR173-5p as well as some known* A. thaliana *extracellular vesicle-loaded TAS-derived secondary siRNAs are upregulated during early* B. cinerea *infection.*

Figure 5.TIFF

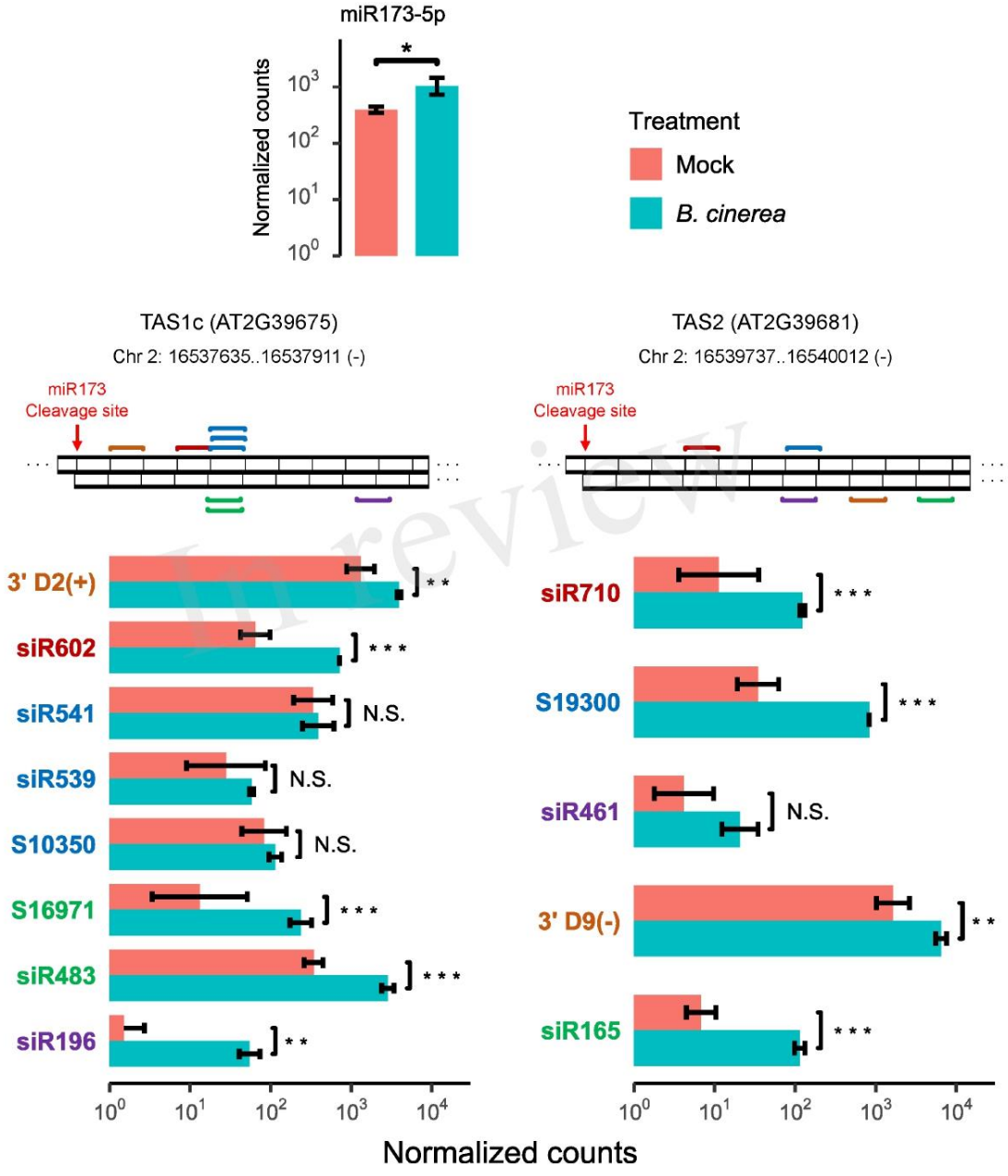
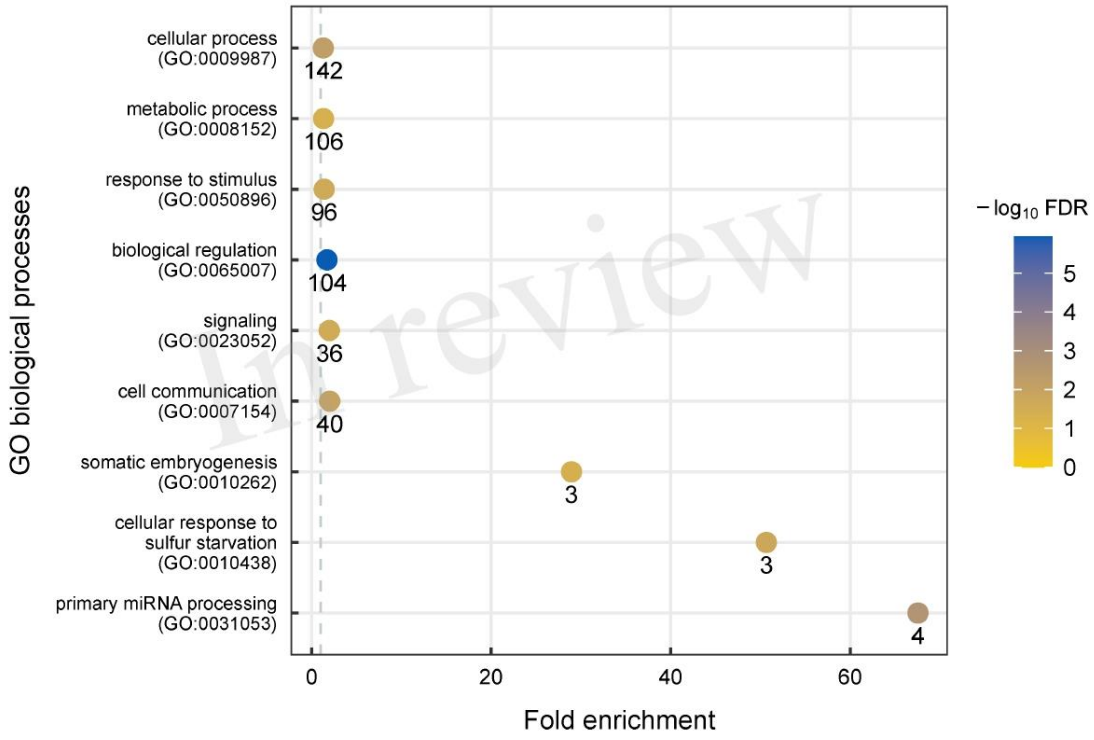


Figure 6. *Biological processes in which the putative mRNA targets are involved.*

Figure 6.TIFF



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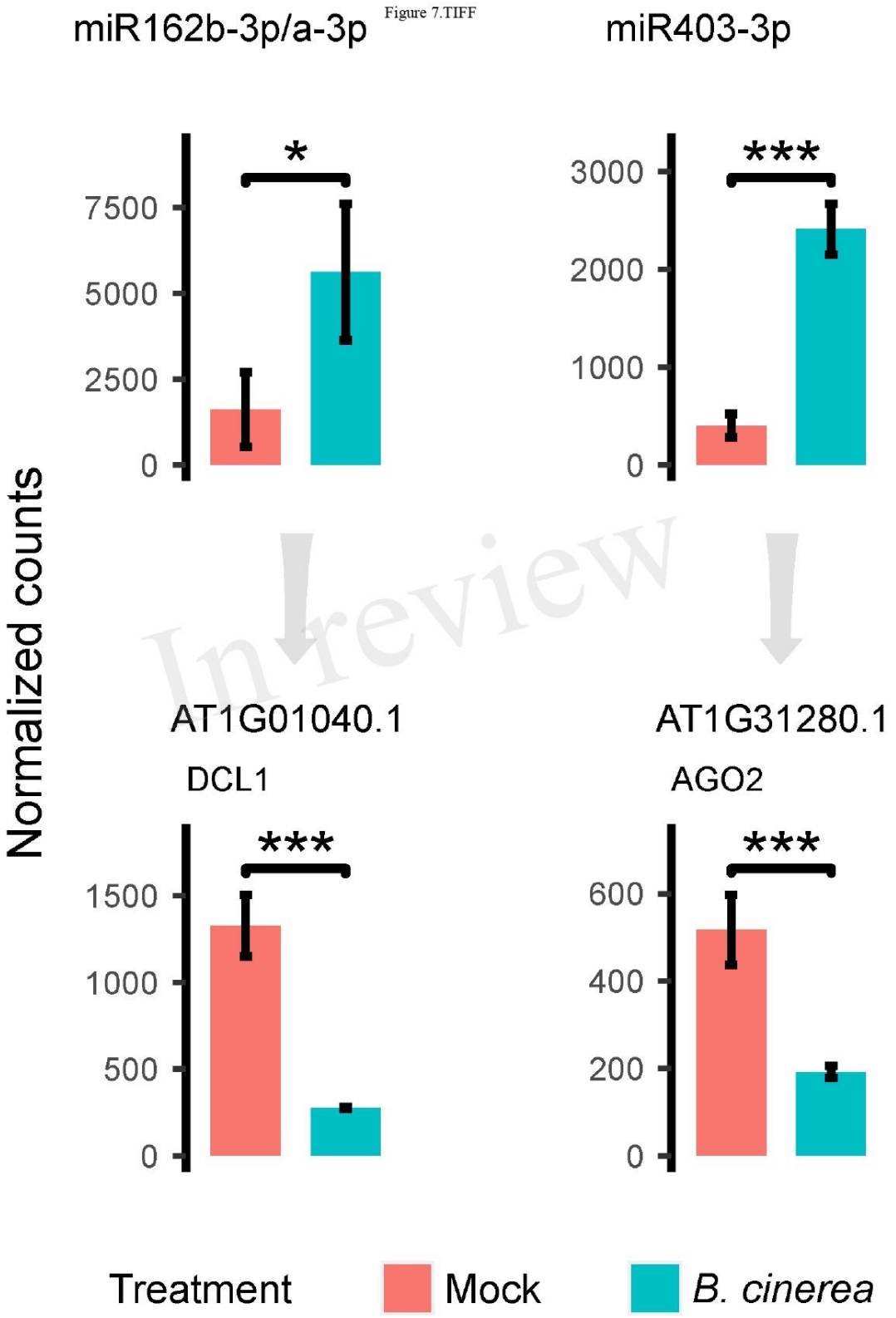

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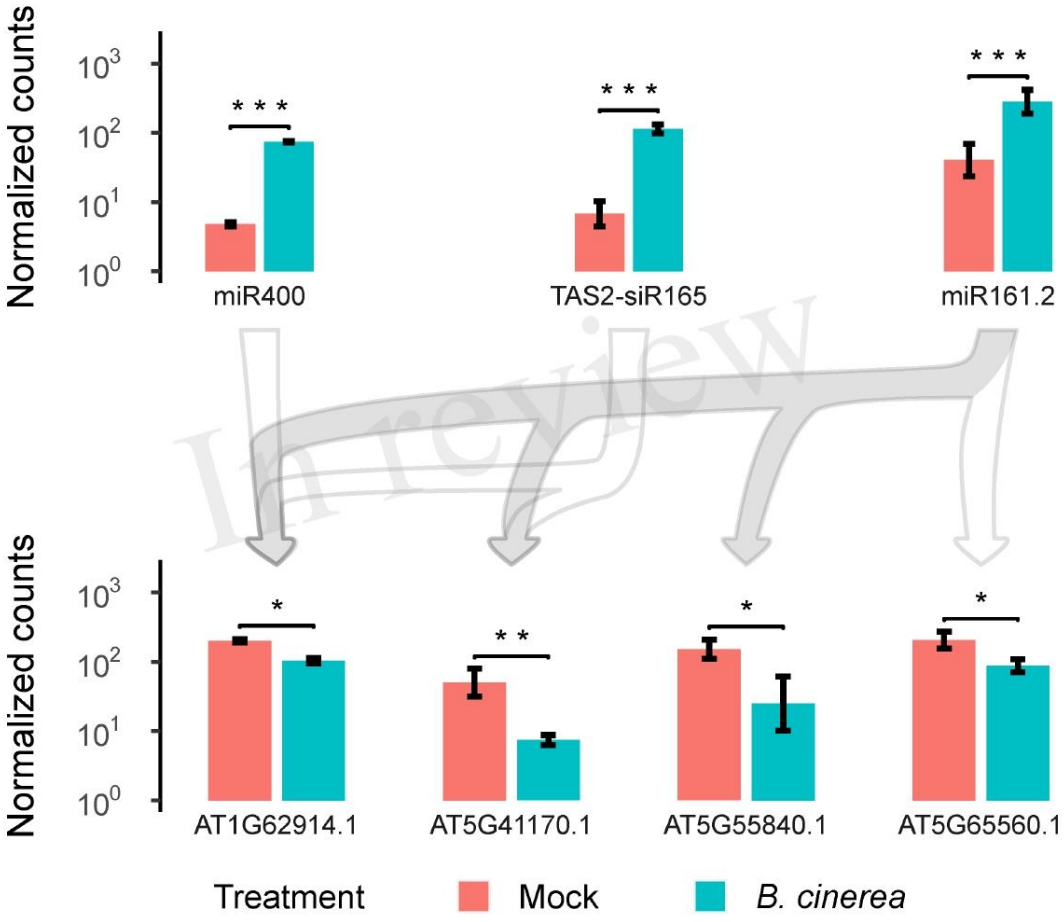

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Supplementary Material

Supplementary Figures and Tables

Supplementary Tables

Supplementary Table 1. All expressed sRNAs from *A. thaliana* treated with mock or *B. cinerea* 6hpi.

Supplementary Table 2. Differentially expressed sRNAs from *A. thaliana* treated with mock or *B. cinerea* 6hpi.

Supplementary Table 3. All expressed mRNAs from *A. thaliana* treated with mock or *B. cinerea* 6hpi.

Supplementary Table 4. Differentially expressed mRNAs from *A. thaliana* treated with mock or *B. cinerea* 6hpi.

Supplementary Table 5. Integrated data of anticorrelated differentially expressed sRNAs and putative mRNA targets from *A. thaliana* treated with mock or *B. cinerea* 6hpi.

Supplementary Table 6. Enriched GO terms and putative mRNA targets associated with them.

Supplementary Figures

Supplementary Figure 1. Principal Component Analysis for sRNA and mRNA read counts from mock and *B. cinerea* treated samples (6 hpi). There were two replicates for each treatment of Plots for sRNAs and mRNAs (are shown on left and right, respectively), read counts were normalized by *rlog transformation*.

Supplementary Figure 2. Length distribution of mapped reads to the *A. thaliana* genome. There were two replicates from mock and *B. cinerea* treatments.

Supplementary Figure 3. Upregulation of miRNAs involved in the fine-tuning of hormone signaling pathways. The plot shows the expression profile of miR167, miR159, miR319 and their targets between mock and *B. cinerea* treatments. Normalized counts of expression values were obtained using the DESeq2 algorithm. Error bars represent standard deviation of two technical replicates. Differential expression between mock and *B. cinerea* treatments is indicated by asterisks (p-value < 0.05 (*) and < 0.001 (***), or no significant (N.S.)). Arrows connect miRNAs with their target genes.

Supplementary Figure 4. Secondary siRNAs derived from the TAS1c transcript from AT2G39675 locus, biogenesis of which are triggered by miR173. miR173 mediates the cleavage of the TAS1c primary transcript, at the position indicated by the red arrow. This cleavage triggers the dsRNA conversion of the resulting downstream cleaved RNA fragment and the sequential production of the secondary siRNAs at approximated 21 nt phase intervals, starting at the miR173 cleavage site.

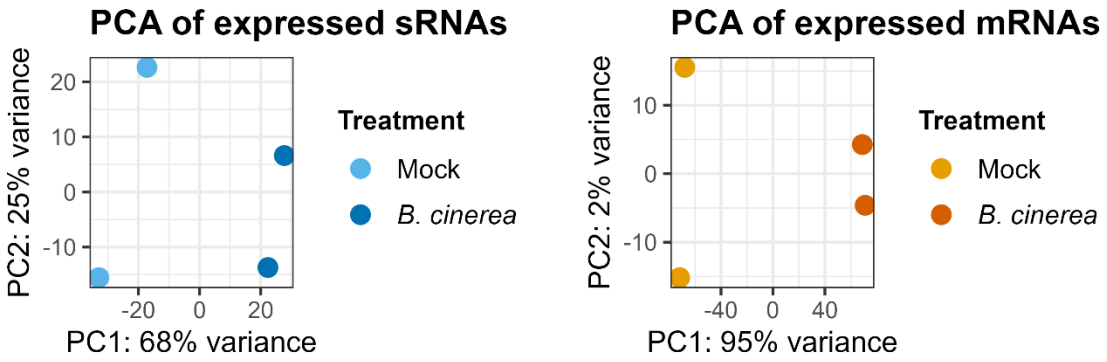
Sequences in color indicate the secondary siRNAs that Cai et al., (2018) described that are loaded in *A. thaliana* extracellular vesicles.

Supplementary Figure 5. Secondary siRNAs derived from the TAS2 transcript from AT2G39681 locus, biogenesis of which are triggered by miR173. miR173 mediates the cleavage of the TAS2 primary transcript, at the position indicated by the red arrow. This cleavage triggers the dsRNA conversion of the resulting downstream cleaved RNA fragment and the sequential production of the secondary siRNAs at an approximated 21 nt phase intervals, starting at the miR173 cleavage site. Sequences in color indicate the secondary siRNAs that Cai et al., (2018) described that are loaded in *A. thaliana* extracellular vesicles.

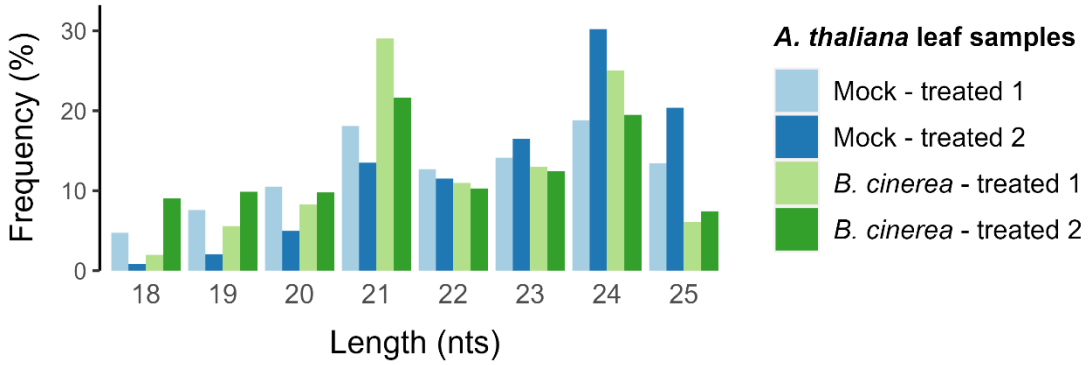
Supplementary Figure 6. Differentially expressed mRNAs during *B. cinerea* infection. Differentially expressed mRNA (p-value ≤ 0.05) indicated in orange, and mRNAs without differential expression in gray. The numbers at the corners indicate upregulated (above) or downregulated (below) mRNAs in *B. cinerea* treatment compared with mock.

Supplementary Figure 7. miR838 is upregulated during *B. cinerea* infection. The plot shows the expression profile of miR838 between mock and *B. cinerea* treatments. Normalized counts of expression values were obtained using the DESeq2 algorithm. Error bars represent standard deviation of two technical replicates. Differential expression between mock and *B. cinerea* treatment is indicated by an asterisk (p-value < 0.05 (*)).

Supplementary Figure 1. *Principal Component Analysis for sRNA and mRNA read counts from mock and* B. cinerea *treated samples (6 hpi).*



Supplementary Figure 2. *Length distribution of mapped reads to the* A. thaliana *genome.*



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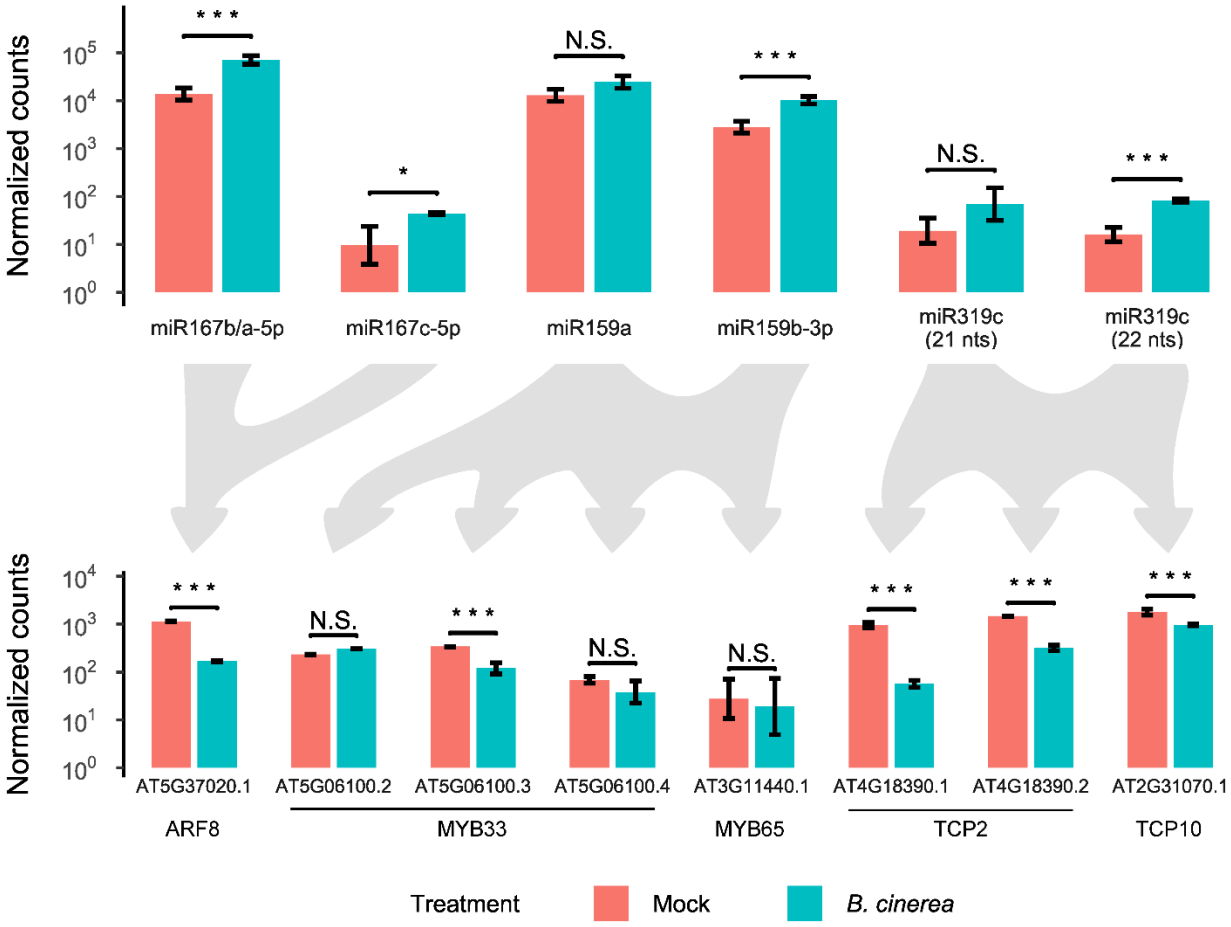

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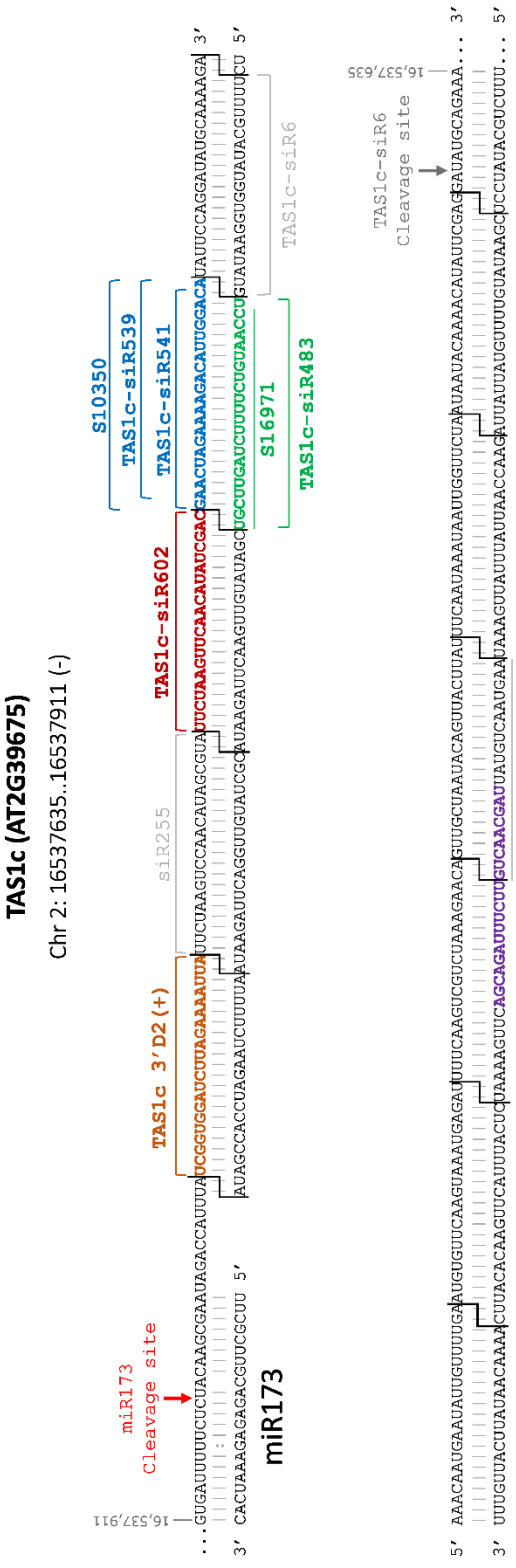

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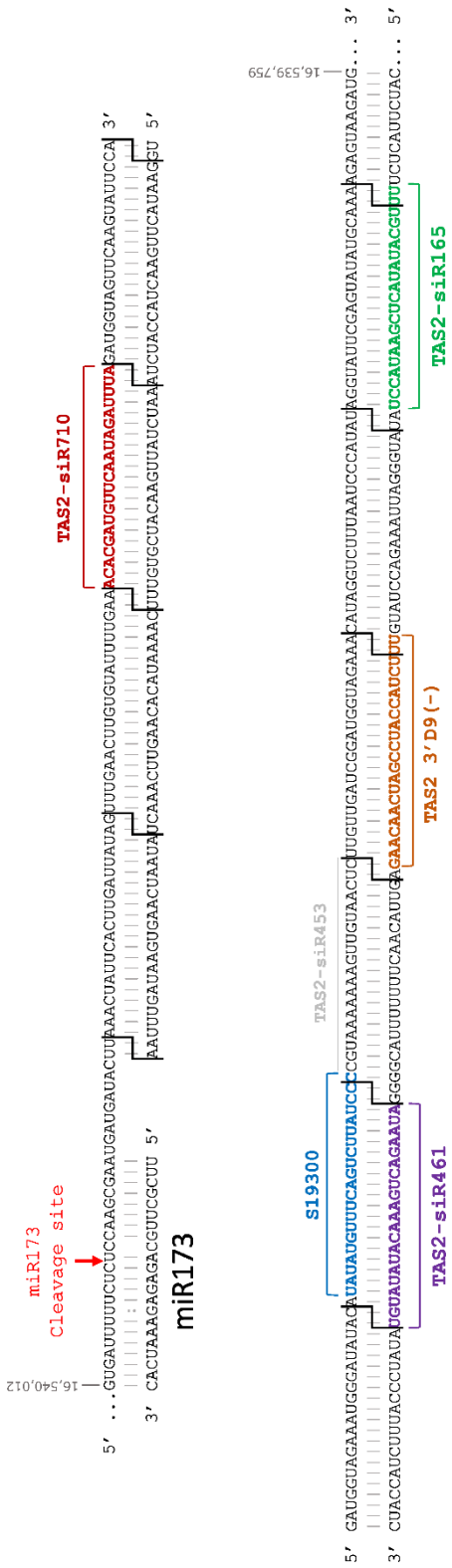
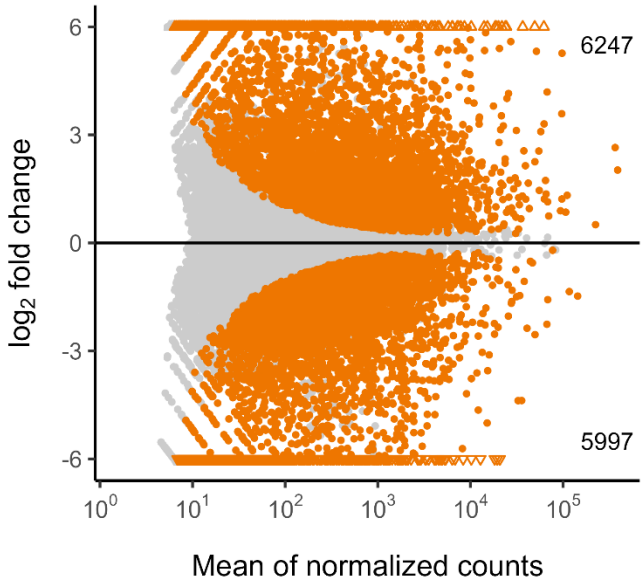
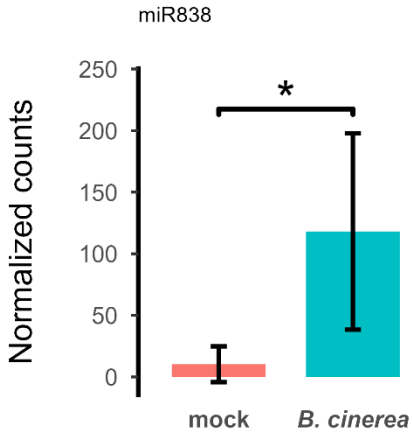


Supplementary Figure 6. *Differentially expressed mRNAs during* B. cinerea *infection.*



Supplementary Figure 7. *miR838 is upregulated during* B. cinerea *infection.*



Discusión

Dado que *Botrytis cinerea* (Botrytis) es un hongo que ocasiona la pérdida de numerosos cultivos importantes para el consumo humano, es necesario comprender los mecanismos que regulan la interacción con sus plantas hospederas para el desarrollo de estrategias para su contención. El objetivo de este trabajo fue el de identificar RNAs pequeños (sRNAs) regulatorios de la interacción de este hongo con la planta modelo *Arabidopsis thaliana* (Arabidopsis), específicamente durante tiempos tempranos de la interacción. Para ello, comparé los niveles de acumulación de sRNAs de hojas de plantas de cuatro semanas tratadas con Botrytis o con tratamiento control (mock) a las 6 horas después de la inoculación (hpi).

Ausencia de sRNAs de Botrytis a las 6 hpi

Weiberg et al. (2013) analizaron bibliotecas de sRNAs extraídos de muestras de hojas de Arabidopsis tratadas con Botrytis [$2x10^5$ esporas/mL] a las 24, 48 y 72 hpi y reportaron que sRNAs de Botrytis participan como efectores en la interacción con Arabidopsis, reprimiendo genes de defensa de la planta (regulación entre reinos). He et al. (2023) reportaron que vesículas extracelulares de Botrytis (del tipo de vesículas que transporta a los sRNAs efectores de este hongo) se encuentran presentes en el sitio de la infección en hojas de Arabidopsis, según análisis de microscopia que realizaron a las 10 hpi. Para este trabajo, extrajimos sRNAs de muestras de hojas de Arabidopsis tratadas con solución control o con Botrytis a 6 hpi, utilizando una concentración de $5x10^4$ esporas/mL, tal como se ha descrito anteriormente (L'Haridon et al., 2011).

Para esta etapa temprana de la infección no hubo acumulación de estos sRNAs conocidos de Botrytis que participan en la regulación entre reinos. Esto pudiera ser debido a que 6 hpi es un tiempo muy temprano en la interacción en el que Botrytis aún no ha producido esos sRNAs o no en la suficiente cantidad como para ser detectados y/o quizás también debido a la menor concentración de esporas inoculadas con respecto del trabajo de Weiberg et al. (2013). Es importante, entonces, estudiar cómo es que diferencias en las condiciones iniciales de inoculación afectan el crecimiento y desarrollo del hongo, así como el progreso de la infección. En general, esto resalta la importancia de llevar un seguimiento fino de la temporalidad y espacialidad

(particularmente durante etapas tempranas) de la infección en la investigación de los diferentes mecanismos moleculares que participan en esta interacción.

Diferentes clases de sRNAs de Arabidopsis son sensibles a la infección causada por Botrytis

En este trabajo, se mostró que diferentes clases de sRNAs (hc-siRNAs, miRNAs, siRNA secundarios, tsRNAs y otros posibles hpRNAs) presentan diferencias en su expresión en presencia de Botrytis en comparación con el tratamiento control. Parece haber importantes diferencias entre las proporciones de las clases de todos los sRNAs expresados no redundantes con los subconjuntos diferencialmente expresados, tanto inducidos como reprimidos (Figuras 1 y 2 del Artículo). Los datos sugieren que la interacción temprana entre Botrytis y Arabidopsis se caracteriza por la inducción de miRNAs y la represión de hc-siRNAs.

Dado que los hc-siRNAs principalmente ejercen su regulación a nivel transcripcional, no se realizó una búsqueda para éstos de posibles genes blancos regulados a nivel postranscripcional. Estos sRNAs regulan la metilación del DNA en regiones repetitivas o con transposones en el genoma, la represión de estos sRNAs podría significar desmetilación de dichas regiones, lo que podría conducir a una mayor facilidad de expresión de genes relacionados con la defensa debido a la relajación de la estructura de la cromatina (Hannan Parker et al., 2022). Los hc-siRNAs representaron una proporción importante de los sRNAs no redundantes expresados diferencialmente a las 6 hpi, lo que representa el 65% de los sRNAs reprimidos con el tratamiento con Botrytis en comparación con el tratamiento control. Esto sugiere que la represión temprana de hc-siRNAs en Arabidopsis podría ser un mecanismo importante durante la interacción con Botrytis, facilitando en la planta la expresión de genes relacionados con la defensa. Se requiere más investigación sobre los tipos de loci de los que provienen estos hc-siRNAs y de sus ubicaciones en el genoma, principalmente para determinar si en la vecindad de los contextos genómicos hay genes relacionados con la inmunidad vegetal que pudieran estar inducidos. También sería bastante valioso contar con información de la metilación del DNA en estos loci para ver si efectivamente hay una desmetilación en estas regiones.

Los miRNAs no solamente fueron la clase más representada de sRNAs no redundantes inducidos, sino que también fue la clase con mayores niveles de acumulación en presencia de Botrytis (Figuras 2 y 3 del Artículo). El perfil de inducción de miRNAs en presencia del hongo sugiere que esta es una respuesta de la planta que favorece la represión de reguladores negativos

de la inmunidad. En este trabajo se mostró que a las 6 hpi con Botrytis, Arabidopsis induce algunos sRNAs conocidos por ser sensibles a patógenos, que incluyen tanto a miRNAs como a siRNAs secundarios y que también son parte de los sRNAs más acumulados (Figuras 3 y 4 del Artículo). Llama la atención el hecho de que la mayoría de los miRNAs más abundantes son, de hecho, miRNAs evolutivamente conservados (Figura 3 del Artículo). Sería interesante investigar si esto ocurre en otras especies vegetales a tiempos tempranos de la interacción con este hongo y si esto también ocurre con otros patógenos. De ser así, sería también importante averiguar si esto representa una oportunidad para desarrollar una estrategia para el control de Botrytis que fuese aplicable en diversas especies vegetales.

Inducción de la expresión de miRNAs reguladores de hormonas vegetales durante etapas tempranas de la infección por Botrytis

La activación de la inmunidad de las plantas también suele correlacionarse con la regulación positiva de miRNAs reguladores de hormonas vegetales, ya que existe una compensación entre el crecimiento de las plantas y las actividades de defensa (Qiao et al., 2021). En este trabajo, se mostró que miR167, miR159 y miR319 estuvieron inducidos en presencia de Botrytis, mientras que genes blancos estuvieron reprimidos (Figura suplementaria 3 del Artículo). Estos miRNAs regulan genes blancos relacionados con las vías de señalización de auxinas, ácido abscísico (ABA) y ácido jasmónico (JA), respectivamente (Reyes & Chua, 2007; Rhoades et al., 2002; Schommer et al., 2008; W. Zhang et al., 2011). Esto sugiere que estos miRNAs podrían estar mediando una respuesta a través de estas vías en Arabidopsis durante la interacción temprana con Botrytis. De acuerdo con la importancia de estos miRNAs en la respuesta al estrés biótico, Zhang et al., (2011) informaron que hojas de Arabidopsis mostraron inducción de miR167 y miR159 a las 6 hpi de haber sido inoculadas con una cepa no virulenta del patógeno bacteriano *Pseudomonas syringae* pv. *Tomato* con un sistema de secreción de tipo III mutado *hrcC*; mientras que hojas inoculadas con una cepa avirulenta portadora de la proteína efectora *avrRpt2,* mostraron inducción de miR159 y miR319 a las 14 hpi. Este grupo de investigación reportó la respectiva represión de genes blancos de estos miRNAs, lo que sugiere la represión de los componentes implicados en las vías de señalización de auxinas, ABA y JA. Por otra parte, Jin y Wu (2015) y Wu et al. (2020) mostraron que sly-miR159 y sly-miR319 y sus respectivos genes blancos también están inducidos y reprimidos, respectivamente en tomate durante la infección por Botrytis, lo cual también resalta la

participación de estos miRNAs en la interacción de las plantas con este hongo fitopatógeno. Ellos también reportaron que líneas de Arabidopsis de sobreexpresión de miR319c mostraron no solo la disminución del gen blanco TCP2, sino también una mayor resistencia a la infección por Botrytis, señalando a TCP2 como un regulador negativo de la resistencia de Arabidopsis a la infección causada por Botrytis.

En conjunto, estos datos sugieren que la regulación de la expresión de miR167, miR159, miR319 y de sus genes blancos es importante durante una etapa temprana de la interacción entre Arabidopsis y Botrytis, probablemente regulando positivamente las respuestas de defensa de las plantas, por lo menos para el caso de miR319.

sRNAs de Arabidopsis que regulan genes entre reinos responden temprano a Botrytis

Cai et al. (2018) mostraron que como parte de los mecanismos de defensa de Arabidopsis, TAS1c-siR483 y TAS2-siR453 (siRNAs secundarios derivados de los transcritos de TAS1c y TAS2) se cargan selectivamente en las vesículas extracelulares de Arabidopsis para ser transportados a células de Botrytis y silenciar genes de virulencia (regulación entre reinos). Este grupo de investigación también reportó que TAS1c-siR483, TAS2-siR453, IGN-siR1 (un hc-siRNA) y miR166 se localizaron dentro de protoplastos de Botrytis purificados de hojas de Arabidopsis infectadas con este hongo. He et al. (2023) reportaron que vesículas extracelulares, del tipo que puede portar a estos sRNAs de Arabidopsis, ya están presentes en el sito de infección a las 10 hpi. Zhang et al., (2016) informaron que los miRNAs de algodón miR159 y miR166 también pueden inducir un silenciamiento entre reinos de genes relacionados con la virulencia, en este caso, con el patógeno fúngico *Verticillium dahliae*. Este grupo mostró que estos dos miRNAs estaban presentes dentro de las células fúngicas y que se inducían tras la infección y que sus posibles genes blancos en el hongo, HiC-15 y Clp-1, estaban justamente reprimidos en las hifas de éste. En este trabajo se mostró que TAS1c-siR483, miR166e-3p/f/b-3p/a-3p/g/c/d/h/i y miR159b-3p se inducen y son parte de los sRNAs más abundantes, en hojas de Arabidopsis en tiempos tempranos de la interacción con Botrytis, (Figura 3 del Artículo).

Estos datos sugieren que la inducción de los sRNAs transportados por vesículas extracelulares de plantas, TAS1c-siR483 y posiblemente miR166 y miR159, es importante en la respuesta temprana ante Botrytis. Podría ser interesante investigar una participación temprana de regulación entre reinos mediado por estos sRNAs de Arabidopsis.

Regulación de DCL1 y AGO2 mediada por miRNAs como parte de la respuesta temprana a Botrytis

Para este trabajo se realizó un análisis de enriquecimiento de términos GO asociados a los posibles genes blancos de los sRNAs expresados diferencialmente que ejercen sus funciones a nivel postranscripcional. Este análisis reveló que los genes blancos están involucrados en diferentes procesos biológicos. En particular, se reportó que hubo un alto enriquecimiento para el término GO "primary miRNA processing (GO:0031053)". Uno de los genes asociados a este término fue DCL1, cuya proteína está involucrada en la biogénesis de miRNAs (Morgado, 2020) y cuyo transcrito es regulado por miR162 (Xie et al., 2003). Zhang et al. (2015) reportaron que cuando plantas de arroz son infectadas con el patógeno *Magnaporthe oryzae* hay una represión de OsDCL1 e inducción de osa-miR162a. Este grupo de investigación también reportó que líneas de arroz de RNAi de OsDCL1 expresaban constitutivamente genes relacionados con la defensa y también eran más resistentes a cepas virulentas de *M. oryzae*, planteando la hipótesis de un papel negativo de OsDCL1 en la inmunidad del arroz. En este trabajo se mostró que en una etapa temprana de la infección causada por Botrytis, miR162b-3p/a-3p se encuentra inducido, mientras que su gen blanco, DCL1, se encuentra reprimido (Figura 7 del Artículo). Rajagopalan *et al.*, (2006) reportaron que además de miR162, miR838 es otro miRNA con potencial para regular DCL1 a nivel postranscripcional. Este grupo reportó que este miRNA deriva de una estructura tipo tallo-asa dentro del intrón 14 del pre-mRNA de DCL1 y propusieron que la presencia de este miRNA intrónico permite un mecanismo de autorregulación que ayuda a mantener la homeostasis de DCL1 en Arabidopsis. En este trabajo mostré que miR838 se encuentra inducido en el tratamiento con Botrytis (Figura suplementaria 7 del Artículo).

AGO2 fue otro ejemplo de un gen relacionado con el silenciamiento guiado por sRNAs que estuvo asociado con un término GO enriquecido, en este caso el término fue "response to stimulus (GO:0050896)". Allen et al. (2005) reportaron que miR403-3p regula negativamente a AGO2. Harvey et al. (2011), por otra parte, han caracterizado a AGO2 como un gen de defensa antiviral que responde a varios virus de plantas. Este grupo de investigación menciona que AGO1 representa una primera capa de defensa mediada por sRNAs en las interacciones entre plantas y virus, que puede ser inactivada por algunos virus que producen supresores de AGO1. También mencionan que esto activa una segunda capa de defensa mediada por sRNAs donde AGO2 ya no es reprimido por miR403 que se asociaba con AGO1. En este trabajo se mostró que miR403-3p

estaba inducido y que AGO2 estaba reprimido en la infección temprana por Botrytis (Figura 7 del Artículo).

En conjunto, estos resultados sugieren que la represión de DCL1 y AGO2 podría ser importante en una etapa temprana de interacción de Arabidopsis con Botrytis. Dado que se ha hipotetizado un rol negativo para DCL1 en la inmunidad en arroz, es posible que su represión en Arabidopsis a tiempos tempranos de interactuar con Botrytis sea un mecanismo que regule positivamente las defensas de la planta. A pesar de la represión temprana de DCL1, se muestra el aumento de la acumulación de diversos miRNAs (Figura 2 del Artículo) de Arabidopsis en presencia de Botrytis. Esto podría indicar que otros mecanismos reguladores y no solo la transcripción de DCL1, actúan para promover la actividad de la enzima DCL1 para lograr la acumulación de los miRNAs. Recordando que Arabidopsis de genotipo silvestre son al final sensibles a Botrytis, para el caso de la represión de AGO2, bien podría tratarse de una regulación inducida por el hongo que más bien es perjudicial para la planta. Podría ser una estrategia encaminada a silenciar una capa de inmunidad mediada por sRNAs en la planta.

Silenciamiento de genes PPR como respuesta temprana a la infección causada por Botrytis

Asociado con el término GO enriquecido “biological regulation (GO:0065007)”, se encontró a un gen de la familia *pentatricopeptide repeat-containing protein* (PPR) (AT5G55840). Como parte de su proyecto de maestría, mi compañera Ana Karen Ávila, al analizar el degradoma de raíces de Arabidopsis en interacción con *Serendipita indica*, un hongo con el que Arabidopsis podría formar una asociación benéfica, encontró que miR161.2 regula negativamente a transcritos de genes de la familia PPR. Ella mostró que miR161.2 media el silenciamiento por corte de este gen PPR, AT5G55840 (Ana Karen Ávila-Sandoval, comunicación personal, junio, 2023). Por otro lado, Hou et al., (2019) reportaron que miR161 regula positivamente la defensa de Arabidopsis contra *Phytophthora capsici*, causando la producción de siRNAs secundarios a partir de transcritos de genes PPR. Estos siRNAs secundarios, a su vez, potencialmente regulan genes de virulencia de este oomiceto patógeno (regulación entre reinos), pues se pueden encontrar en vesículas extracelulares de la planta (Hou et al., 2019). Ellos también mostraron que miR161 y los siRNAs secundarios derivados de los PPR, PPR-siRNA-1 y PPR-siRNA-2 estaban inducidos tras la exposición a *P. capsici* y que líneas de sobreexpresión y *knock-out* de Arabidopsis de *MIR161* muestran una mayor resistencia o mucha mayor susceptibilidad a *P. capsici*, respectivamente. Este

grupo mostró que líneas mutantes de Arabidopsis de RDR6 y SGS3 (enzimas implicadas en la producción de siRNAs secundarios) también eran mucho más susceptibles al patógeno, sugiriendo que la vía de siRNAs secundarios tiene un papel importante en la defensa de las plantas durante la infección por *P. capsici*. Además de la validación de la regulación del PPR AT5G55840 (Ana Karen Ávila-Sandoval, comunicación personal, junio, 2023), se ha confirmado previamente la regulación de los genes PPR AT1G62914 y AT5G41170 por miR161.2 (Allen et al., 2004; Vargas-Asencio & Perry, 2020). En el artículo sometido a la revista Frontiers in Genetics se presentó otro gen blanco de la familia PPR, AT5G65560, que posiblemente es regulado por miR161.2 (Figura 8 y Tabla suplementaria 5 del Artículo). Se requiere de trabajo experimental para validar esta regulación.

Además de miR161.2, otros sRNAs que pueden regular genes PPR de Arabidopsis son miR161.1, miR400 y algunos siRNAs secundarios derivados de transcritos de los loci TAS1a/b/c y TAS2 (Allen et al., 2004; Howell et al., 2007; Park et al., 2014). Por ejemplo, Park et al. (2014) reportaron que miR400 regula el gen PPR AT1G62720 y mutantes knockdown de este gen PPR son más susceptibles a Botrytis. El PPR AT1G62914 además de ser regulado por miR161.2, potencialmente también es regulado tanto por miR400 como por TAS2-siR165; mientras que el PPR AT5G41170 es también potencialmente regulado por TAS2-siR165 (Figura 8 y Tabla suplementaria 5 del Artículo). Para este trabajo se mostró que en plantas tratadas con Botrytis hay una inducción temprana de miR161.2, miR400 y TAS2-siR165 (Figuras 5 y 8 del Artículo). A pesar de que no hubo diferencia en la acumulación de AT1G62720 (el PPR blanco validado para miR400) entre los tratamientos, en este trabajo se mostró que los PPRs AT1G62914, AT5G41170, AT5G55840 y AT5G65560 sí se encuentran reprimidos en presencia de Botrytis (Figura 8 del Artículo). Es importante mencionar que la producción de siRNAs secundarios derivados de transcritos TAS es desencadenada por el corte de estos transcritos mediad por miR173, el cuál también muestra una inducción en presencia de Botrytis (Figura 5 del Artículo). Estos resultados sugieren que miR161.2, miR400 y miR173/TAS2-siR165 median la regulación de un subconjunto de genes PPR durante la infección temprana por Botrytis en Arabidopsis. Investigaciones futuras podrían ayudar a indicar si siRNAs secundarios son producidos a partir de estos PPRs regulados, los cuales pudieran incluso ser parte de la regulación entre reinos.

Conclusiones

Los resultados que se presentan en esta tesis confirman que sí hay RNAs pequeños (sRNAs) que participan en la regulación de la interacción de *Arabidopsis thaliana* (Arabidopsis) con el hongo fitopatógeno *Botrytis cinerea* (Botrytis). El hecho de que una proporción importante de sRNAs reprimidos en presencia de Botrytis sean hc-siRNAs sugiere que una de las respuestas tempranas de las plantas se orienta a mantener una estructura relajada de la cromatina, lo cual podría permitir una transcripción más eficiente de genes de defensa. Otros análisis se requieren para comprobar esta hipótesis.

La inducción de miRNAs involucrados en la regulación de hormonas vegetales en presencia de Botrytis (que pudieran estar reprimiendo posibles reguladores negativos de la respuesta inmune), la inducción de algunos sRNAs que participan en la regulación de genes entre reinos (posible preparación de la planta para enviar estos sRNAs en vesículas extracelulares a las células de Botrytis) y la inducción de sRNAs que desencadenan la producción de siRNAs secundarios a partir de transcritos PPR, podrían representar respuestas tempranas que regulan positivamente la inmunidad en plantas contra la infección causada por Botrytis.

Durante tiempos tempranos de la interacción entre Arabidopsis y Botrytis, los niveles de expresión de DCL1 y de AGO2, componentes clave de las rutas de silenciamiento mediadas por sRNAs, son regulados precisamente por sRNAs, lo cual podría ser importante en una etapa temprana de la interacción entre Arabidopsis y Botrytis. Más investigación se requiere para entender esta regulación.

Perspectivas

Dado que reportamos varios sRNAs que potencialmente participan en la regulación temprana de la interacción de *Arabidopsis thaliana* (Arabidopsis) con el hongo fitopatógeno *Botrytis cinerea* (Botrytis), será bastante valioso tener validaciones experimentales de los patrones de expresión de dichos sRNAs y de sus genes blanco en esta etapa de la interacción, para consolidar estos hallazgos.

En vista de que una buena proporción de los sRNAs diferencialmente expresados en presencia de Botrytis corresponden a hc-siRNAs, resulta de interés averiguar el papel de estos sRNAs en la regulación de la interacción Arabidopsis-Botrytis.

Queda pendiente extender los análisis de esta tesis para más tiempos de la interacción, con el fin de dar un seguimiento a las dinámicas de expresión de los sRNAs reguladores y de sus genes blancos, o bien, para identificar nuevas regulaciones.

Sería interesante extender los análisis presentados en este trabajo para genotipos de Arabidopsis resistentes a la infección por Botrytis y contrastar con los datos aquí presentados con el objetivo de identificar otros sRNAs, así como a sus genes blanco, que pudieran estar involucrados en la regulación temprana de la interacción.

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