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IDENTIFICACIÓN Y CARACTERIZACIÓN DE LOS ELEMENTOS MOLECULARES QUE PARTICIPAN EN LA INMUNIDAD INNATA VEGETAL INDUCIDA POR MONÓMEROS DE CUTINA

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Abreviaturas

ABA: Ácido abscísico
cDNA: Ácido desoxirribonucleico complementario
DAMP: Patrones moleculares asociados a daño
EMS: Etil-metano-sulfonato
ET: Etileno
ETI: Inmunidad activada por efectores
FA: Frecuencia alélica
FAS: Ácido graso sintasa
gADN: Ácido desoxirribonucleico genómico
GUS: β-glucoronidasa
HR: Respuesta hipersensible
JA: Ácido jasmónico
LACS: Acil-CoA sintetasa de ácidos grasos de cadena larga
LCFA: Ácidos grasos de cadena larga
LTP: Proteína de transferencia de lípidos
MAPK: Proteína cinasa activada por mitógenos
PAMP: Patrones moleculares asociados a patógenos
PCD: Muerte celular programada
PME: Pectin-metil-esterasa
PRR: Receptores de reconocimiento de patrones
PTI: Inmunidad activada por patrones
RE: Retículo endoplásmico
ROS: Especies reactivas de oxígeno
SA: Ácido salicílico
SEM: Microscopía electrónica de barrido
SNP: Polimorfismo de un solo nucleótido
TAIR: Del inglés, *The Arabidopsis Information Resource*
TEM: Microscopía electrónica de transmisión
VLCFA: Ácidos grasos de cadena muy larga
wt: tipo silvestre

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Resumen.

La cutícula es una capa hidrofóbica que cubre las células epidérmicas de las partes aéreas de las plantas y constituye la primera línea de defensa contra patógenos. Está formada principalmente por cutina y ceras. Dado a la importancia de sus componentes, este trabajo doctoral se centra en profundizar el entendimiento a nivel molecular, del papel que desempeñan en la activación de la inmunidad innata en *Arabidopsis thaliana* en presencia del hongo *Botrytis cinerea.* A través de un análisis fisiológico y transcriptómico, en mutantes con defectos cuticulares y alteradas en el contenido de los componentes de ceras y/o de monómeros de cutina; se determinó que todas mostraron una mayor permeabilidad de la cutícula y acumulación de especies reactivas de oxígeno; sin embargo, solo las afectadas en el contenido de cutina mostraron resistencia hacia el hongo. A través del análisis transcriptómico se demostró que, la inmunidad en las mutantes afectadas en cutina es independiente de la expresión de genes canónicos relacionados con la defensa y de genes relacionados con vías de hormonas vegetales, como JA/ET, SA y ABA reportados en la interacción planta-*Botrytis*. Sorprendentemente, se identificó un conjunto de genes implicados en la integridad de la pared celular, entre ellos *AtPME17* y *AtPME41*; así como miembros de la familia de factores de transcripción (AP2/ERF) como *RAP2.6*/*ERF108* y *RAP2.6L*/*ERF113*.

Por otra parte, con el uso de la herramienta computacional SHOREmap se realizó un mapeo *in silico* preliminar, para identificar genes candidatos responsables del fenotipo de la mutante *eca2*, afectada en la estructura de la cutícula y en su contenido de monómeros cutina y en componentes de ceras. El análisis de los datos de la secuenciación masiva de gDNA, de una población de mapeo generada de la cruza entre *eca2* (ecotipo C24) y la planta de tipo silvestre Col-0; identificó una región genómica en el brazo superior del cromosoma 1. Los genes ubicados en esa región, y con mayor probabilidad de causar la mutación en *eca2* fueron, AT1G16110 (*WAKL6*) un gen que codifica para una tipo cinasa asociada a la pared celular; AT1G14520 (*MIOX1*) codifica para una *myo*-inositol oxigenasa, y AT1G12630 que codifica para un factor de transcripción de la familia ERF/AP2. Esta es una primera aproximación; sin embargo, estos genes necesitan ser evaluados para confirmar el fenotipo observado.

Abstract.

The cuticle is a hydrophobic layer that covers the epidermal cells of the aerial parts of plants and constitutes the first line of defense against pathogens. It is mainly made up of cutin and waxes. Given the importance of its components, this study focuses on deepening the understanding of the role they play in the activation of innate immunity in *Arabidopsis thaliana* in the presence of the fungus *Botrytis cinerea*. Through a physiological and transcriptomic analysis, in mutants with cuticular defects and altered content of wax components and/or cutin monomers; it was determined that all showed an increased permeability of the cuticle and accumulation of reactive oxygen species; however, only those affected in the cutin content showed resistance to the fungus. The transcriptomic analysis showed that immunity in mutants affected in cutin is independent of the expression of canonical genes related to defense and genes related to signaling pathways of hormones, such as JA/ET, SA and ABA reported in the interaction plant-*Botrytis*. Surprisingly, a set of genes involved in cell wall integrity was identified, including *AtPME17* and *AtPME41*; as well as members of the family of transcription factors (AP2/ERF) such as *RAP2.6*/*ERF108* and *RAP2.6L*/*ERF113*.

On the other hand, with the use of the computational tool SHOREmap, a preliminary *in silico* mapping was carried out to identify candidate genes responsible for the mutation in *eca2*, affected in the structure of the cuticle and in its cutin monomer content and wax components. Analysis of massive gDNA sequencing data from a mapping population generated from the cross *eca2* (ecotype C24) and the wild-type plant Col-0; identified a genomic region on the upper arm of the chromosome 1. The genes located in that region, and most likely to cause the *eca2* phenotype, were AT1G16110 (*WAKL6*), a gene that codes for a cell wall-associated kinase; AT1G14520 (*MIOX1*) codes for a *myo*-inositol oxygenase, and AT1G12630 that codes for a transcription factor of the ERF/AP2 family. This is a first approximation; however, these genes need to be validated to confirm the observed phenotype.

1. Introducción general.

Las plantas están expuestas constantemente a condiciones ambientales adversas y en comunicación recíproca con insectos, nematodos, virus, bacterias y hongos tanto benéficos como patógenos, durante las distintas etapas de su desarrollo (Dangl et al., 2013). Uno de los primeros sitios donde las plantas interaccionan con los fitopatógenos, es la cutícula, por lo que es considerada la primera línea de defensa contra éstos. Recientemente se ha descrito que los componentes químicos de la cutícula como la cutina y las ceras, regulan íntimamente no solo el desarrollo y defensa de las plantas, sino también el crecimiento de los fitopatógenos (Aragón et al., 2017).

En la interacción planta-fitopatógenos, las respuestas de defensa pueden derivarse de las barreras preformadas como la cutícula, o inducirse en cada una de sus células que forman parte del sistema inmune innato vegetal (Jones y Dangl, 2006; Boller y Felix, 2009). Las plantas han desarrollado una compleja y robusta adaptación para reconocer y/o percibir tanto señales propias, como exógenas, para activar mecanismos de defensa. La activación de respuestas de defensa depende del reconocimiento específico del tipo de patógeno, por medio de un diálogo molecular entre los organismos involucrados. Se han descrito dos principales niveles de defensa. El primero, implica la percepción y reconocimiento de moléculas o elicitores altamente conservadas y específicas de microorganismos patógenos, denominadas de manera general como **PAMP** (del inglés, *Pathogen-associated molecular patterns*), a través de receptores **PRR** (del inglés, *Pattern recognition receptors*), y de moléculas propias de la planta liberadas tras el ataque del fitopatógeno denominadas **DAMP** (del inglés, *Damage-associated molecular patterns*), entre ellas, monómeros de cutina y/o fragmentos de pared celular; las cuales activan el mecanismo de defensa denominado **PTI** (del inglés, *PAMP-triggered immunity*) (Boller y Felix, 2009; Macho y Zipfel, 2014).

Un segundo nivel de defensa puede originarse con patógenos que evaden la **PTI** y ocurre típicamente de manera específica entre planta-patógeno, en el cual proteínas de virulencia producidas por el patógeno (llamadas efectores), son reconocidas por receptores intracelulares del tipo **NLR** (del inglés, *Nucleotide-binding leucine-rich repeat*) y activan la inmunidad activada por efectores, llamada **ETI** (del inglés, *Effector-*

triggered immunity). El reconocimiento intracelular de los efectores se asocia a menudo con la muerte celular programada, **PCD** (del inglés, *Programmed Cell Death*), conocida como respuesta hipersensible, **HR** (del inglés, *Hypersensitive Response*), y la posterior inducción de la resistencia sistémica adquirida, **SAR**, (del inglés, *Systemic Acquired Resistance*) (Jones y Dangl, 2006; Hacquard et al., 2017). Inmediatamente después de la percepción de **PAMP**/**DAMP**/efectores, una respuesta temprana es la producción de especies reactivas de oxígeno (**ROS**), seguido de cascadas de señalización que implican flujos de Ca^{2+} y cascadas de MAP cinasas, **MAPK** (del inglés, *Mitogen-Activated Protein Kinases*), deposición del polisacárido calosa para reforzar la pared celular y producción de fitoalexinas. Todas estas señales convergen en la producción de hormonas vegetales, entre ellas etileno (**ET**), ácido jasmónico (**JA**), ácido salicílico (**SA**) y ácido abscísico (**ABA**), que desencadenan una reprogramación transcripcional de genes relacionados con la defensa (Göhre y Robatzek, 2008; Dodds y Rathjen, 2010).

En los últimos años, se ha descrito que mutantes afectadas en el contenido de cutina, presentan un síndrome defensivo en contra del hongo necrótrofo *Botrytis cinerea*, el cual incluye una rápida acumulación de **ROS** y una activación de la inmunidad innata. En un intento de explicar este síndrome, se propuso que, debido al aumento en la permeabilidad de la cutícula, moléculas como **PAMP** y **DAMP**, son rápidamente reconocidas por los receptores **PRR**, activando más eficientemente las respuestas de defensa (Serrano et al., 2014). Sin embargo, no se cuenta con información similar relacionada a las mutantes con modificaciones en el contenido de ceras. Con base en esto, en el presente trabajo el objetivo es profundizar en los mecanismos de las respuestas de defensa mediadas tanto por los monómeros de cutina, como por los componentes de las ceras, en la interacción *Arabidopsis thaliana-Botrytis cinerea*.

CAPÍTULO I.
Antecedentes generales.

I.1 Mecanismos de defensa pre-formados.

I.1.1 Composición y estructura de la cutícula.

Una de las primeras barreras con las que se encuentra un fitopatógeno, es la cutícula, la cual es una estructura hidrofóbica que cubre la superficie de las células epidérmicas de los órganos aéreos de las plantas (hojas, tallos, semillas, flores y frutos) (Yeats y Rose, 2013). Sin embargo, recientemente se encontró que la cutícula, también está presente en la cofia de la raíz, **RC** (del inglés, *Root Cap*) durante las primeras etapas de su desarrollo, teniendo como función protectora ante el estrés abiótico al que están expuestas las células iniciales del meristemo apical, contribuyendo así al correcto desarrollo y crecimiento de la raíz (Berhin et al., 2019).

La cutícula representa una de las adaptaciones evolutivas, que le han permitido a las plantas contrarrestar los efectos adversos producidos por factores bióticos y/o abióticos (Pollard et al., 2008). La estructura y composición química de la cutícula varía ampliamente entre diferentes especies de plantas e incluso entre órganos y estados de desarrollo. A pesar de esta variabilidad, todas las cutículas están constituidas principalmente por dos tipos de lípidos, cutina y ceras, además de otro compuestos, como fenoles, flavonoides y polisacáridos (celulosa, hemicelulosa y pectina) (Nawrath, 2006; Yeats y Rose, 2013). Debido a la naturaleza de su composición y a su interacción con componentes de la pared celular, la cutícula puede considerarse como una pared celular cutinizada. Se ha planteado que tal interacción puede darse a través de los componentes de la pectina como los ramnogalacturonanos (RG) de baja ramificación, cuya estructura favorece una asociación polisacárido-lípido. Asimismo, por medio de la celulosa, el cual es un polisacárido anfifílico que estabiliza la emulsión de lípidos a través de interacciones hidrofóbicas (Lindman et al., 2021). Por lo tanto, la interacción a través de enlaces no covalentes entre los polisacáridos y la cutina debe ser suficiente para dar a la cutícula fuerza y flexibilidad necesaria para el desarrollo de los órganos de las plantas (Reynoud et al., 2021) (**Figura 1**).

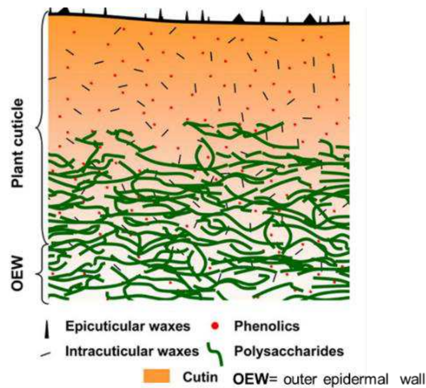

Figura 1. Modelo estructural de la cutícula vegetal, representando la localización de sus diferentes componentes. Imagen adaptada de Domínguez et al*.,* (2011).

El componente estructural mayoritariamente abundante es la cutina (entre el 40 y 80 % de su peso), el cual es un poliéster insoluble constituido por monómeros derivados de ácidos grasos alifáticos de 16 y 18 átomos de carbono (ω-hidroxiácidos, polihidroxi-ácidos y α, ω-ácidos di-carboxílicos (DCA)), además de pequeñas cantidades de glicerol y fenilpropanoides (ácido ferúlico y ácido ρ-cumárico) (Heredia, 2003; Domínguez et al., 2011; Fich et al., 2016) (**Figura 2**).

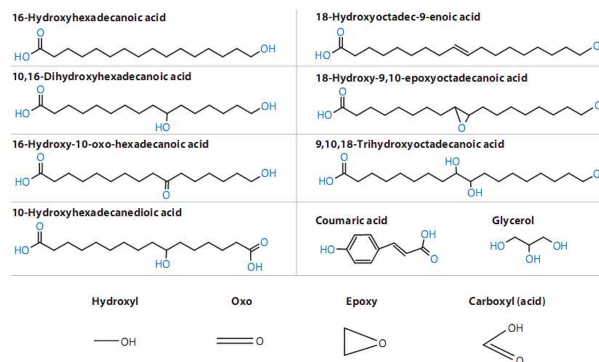

Figura 2. Monómeros de cutina y grupos funcionales típicos que los constituyen. Imagen adaptada de Fich et al., (2016).

El otro componente importante de la cutícula son las ceras, las cuales están localizadas en la superficie (ceras epicuticulares) y embebidas en la matriz de cutina (ceras intracuticulares). Las ceras epicuticulares pueden formar cristales o películas de cera, que a menudo dan un aspecto brillante a la superficie de la cutícula de hojas y de tallos, de algunas especies como *Arabidopsis thaliana* (de ahora en adelante como Arabidopsis) (Samuels et al., 2008; Kunst y Samuels, 2009).

A diferencia de la cutina, las ceras están conformadas por moléculas de carbono no polares, de cadena larga con más de 24 átomos, **VLCFA** (del inglés, *Very Long Chain Fatty Acids*), las cuales son solubles en disolventes orgánicos como el cloroformo. Los componentes típicos de las ceras incluyen hidrocarburos, aldehídos, alcoholes, cetonas y ésteres de ceras (Fernández et al., 2016; Hegebarth y Jetter, 2017) (**Figura 3**).

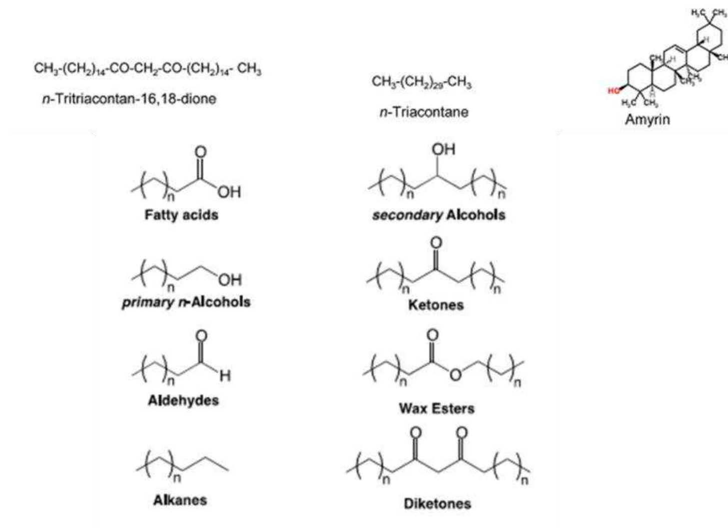

Figura 3. Estructuras químicas de las principales clases de compuestos que componen a las ceras cuticulares. Imagen adaptada de Hegebarth y Jetter (2017).

I.1.2 Biosíntesis de la cutícula.

I.1.2.1 Biosíntesis y polimerización de cutina.

Los pasos básicos de la biosíntesis de la cutícula han sido esclarecidos durante la última década, a través de enfoques de genética directa y reversa en plantas de Arabidopsis y de tomate (*Solanum lycopersicum*) (Beisson et al., 2012; Li-Beisson et al., 2013; Yeats y Rose, 2013; Fich et al., 2016). En las plantas de Arabidopsis, tanto los monómeros de cutina como los componentes de ceras, son derivados de ácidos grasos de cadena larga **LCFA** (del inglés, *Long Chain Fatty Acids*) de C_{16} o C_{18}, que se originan a partir de la síntesis *de novo* de ácidos grasos catalizados por un complejo de ácido graso sintasa, **FAS (**del inglés, *Fatty Acid Synthase*) localizado en los plástidos de las células epidérmicas (Haslam y Kunst, 2013; Yeats y Rose, 2013; Delude et al., 2016). Estos ácidos grasos se transportan al retículo endoplásmico (**RE**), donde se lleva a cabo la síntesis, tanto de monómeros de cutina como de los componentes de ceras, involucrando varias enzimas. En la **Figura 4**, se muestran los pasos esenciales en la biosíntesis de cutina.

Estudios en Arabidopsis han identificado como probable primer paso en la síntesis de cutina, la activación/esterificación de CoA (coenzima-A) a los ácidos grados libres de C_{16} o C_{18} (C_{16}/C_{18}-CoA), de tres acil-CoA sintetasas, **LACS** (del inglés, *Long-chain-Acyl-CoA Synthetase*) LACS1, LACS2, y LACS4 (Lü et al., 2009; Pulsifer et al., 2012). Asimismo, se ha identificado la participación de LACS2 en la biosíntesis de cutina, debido a que su mutante *lacs2*, muestra niveles reducidos de monómeros de cutina (Schnurr et al., 2004; Bessire et al., 2007). De la misma manera, se caracterizó a la mutante *lacs1* de Arabidopsis y se identificó el papel de la enzima LACS1 en la biosíntesis de componentes de ceras cuticulares (Lü et al., 2009). Sin embargo, se ha propuesto que estas dos enzimas parecen tener una redundancia funcional, observada en la doble mutante *lacs1-lacs2*, la cual muestra fenotipos pleiotrópicos, como fusión de órganos, desarrollo anormal de flores y mayor permeabilidad cuticular comparada con las mutantes simples *lacs1* o *lacs2* (Weng et al., 2010).

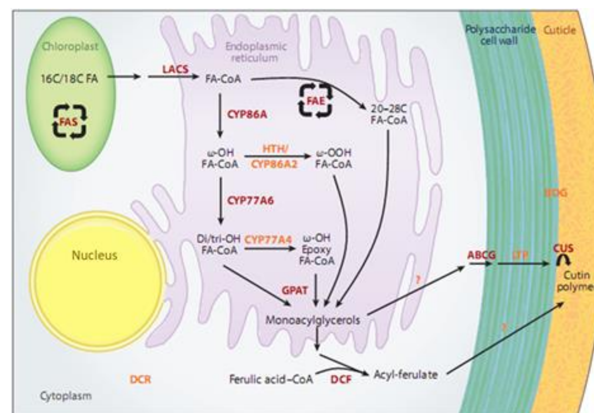

Figura 4. Pasos identificados en la vía de biosíntesis de cutina propuesta en Arabidopsis. Los nombres resaltados en color anaranjado y rojo indican proteínas o complejos de proteínas con una función demostrada. **FA**, ácidos grasos; **FAE**, complejo elongasa de ácidos grasos. Imagen adaptada de Fich et al., (2016).

El siguiente paso en la biosíntesis de cutina, es la oxidación de los precursores. Una vez en el **RE**, los **LCFA** son convertidos en una variedad de ácidos grasos hidro y/o dihidroxi-ácidos (ω-OH, ω-OOH) y epoxi-ácidos, a través de varias reacciones de oxidación catalizadas por enzimas del tipo oxidasas **HTH** (del inglés, *HOTHEAD*) (Kurdyukov et al., 2006a) y de citocromo P450, principalmente miembros de las subfamilias de CYP86A (CYP86A2/ATT1, CYP86A8/LCR y CYP86A4) (Wellesen et al., 2001; Xiao et al., 2004; Li-Beisson et al., 2009) y de CYP77A (CYP77A4 y CYP77A6) (Domínguez

et al., 2015; Fich et al., 2016). Una vez que son sintetizados los ácidos grasos hidroxilados, estos se unen covalentemente a proteínas aciltransferasas intracelulares, como las **GPAT** (del inglés, *Glycerol-3-Phosphate Acyltransferase*) (GPAT4/6/8), para sintetizar precursores de monómeros de cutina (2-monoacil-MAG y diacil-gliceroles-DAG) (Yeats y Rose, 2013; Fich et al., 2016). De manera paralela, se ha identificado la participación de otras aciltransferasas de la familia BAHD localizadas en el citoplasma, que también son esenciales para la formación de la cutina, por ejemplo, **DCR** (del inglés, *Defective in Cuticular Ridges*) y el mutante de pérdida de función del gen (*dcr*) presenta pérdida casi completa de ácidos grasos de C_{16} (principales monómeros de cutina) (Panikashvili et al., 2009); asimismo **DCF** (del inglés, *Deficient in Cutin Ferulate*) que involucra ácido ferúlico para incorporarlo en la cutina (Rautengarten et al., 2012) (ver **Figura 4**).

Para incorporarse a la matriz de la cutina, los monómeros deben exportarse desde el **RE** hacia el sitio de polimerización, a través de la membrana plasmática. Esto fue observado en plantas mutantes (*knock-outs*) de Arabidopsis de los genes que codifican para transportadores de la subfamilia **ABCG** (del inglés, ATP-*binding cassette* G), lo cual ha posibilitado su análisis funcional (especificidad de sustrato, especies químicas transportadas, etc.) (McFarlane et al., 2010). Entre estos están ABCG11, ABCG13 (Bird et al., 2007; Panikashvili et al., 2011) y el ABCG32/PEC1 (Bessire et al., 2011; Fabre et al., 2016; Elejalde-Palmett et al., 2021) implicados en la deposición de cutina. Se ha propuesto otra familia de transportadores, que participan en la deposición de cutina, las proteínas de transferencia de lípidos o **LTP** (del inglés, *Lipid-Transfer-Proteins*), las cuales unen a los precursores de ácidos grasos de cutina, evaluadas bajo condiciones *in vitro* (Debono et al., 2009). Sin embargo, no se ha reportado evidencia bajo condiciones *in vivo* de tal actividad para los monómeros de cutina (Fich et al., 2016), pero si se demostró que LTP1 y LTP2, están involucradas en el transporte de componentes de cera cuticular (Kim et al., 2012) (**Figura 4**).

El último paso en la biosíntesis de cutina es la polimerización de los monómeros, en el cual se han identificado dos rutas, aunque es importante señalar que ninguna de éstas ha sido elucidada por completo. La ruta más conocida se basa en la biosíntesis que

involucra enzimas, mientras que la otra no incluye la participación de éstas, sino que asume la participación de nanoestructuras, que se autoensamblan a partir de precursores de cutina. Dentro de la ruta de biosíntesis que involucra enzimas, en los últimos 2 años, se ha acumulado evidencia sólida que demuestra que las proteínas lipasas/esterasas **GDSL** (Gly-Asp-Ser-Leu motif), son esenciales para la síntesis y deposición de cutina (Domínguez et al., 2015). Aunque la mayoría de las investigaciones sobre la síntesis de cutina se ha realizado en Arabidopsis, estos resultados se derivan del estudio de la cutícula del fruto del tomate. La caracterización de una mutante nula de tomate, *cutin deficient 1* (*cd1*), permitió la identificación y caracterización de una lipasa GDSL extracelular, denominada **CD1**/**CUS1**; cuyos frutos presentaron un incremento en la pérdida de agua debido a una cutícula más delgada (5-10 % del contenido de cutina); y mayor susceptibilidad hacia *B. cinerea*. Asimismo, se demostró bajo condiciones *in vitro*, la preferencia de esta enzima hacia el sustrato 2-mono (dihidroxihexadecanoil) glicerol (2-MHG), principal precursor para la formación de los monómeros de cutina y su participación *in vivo* para su acumulación (Isaacson et al., 2009; Yeats et al., 2012). De manera paralela, en otro estudio usaron ARN de interferencia (**ARNi**) para reducir la expresión del gen *GDSL1* en plantas de tomate y observaron una cutícula con menor grosor y disminución del contenido de cutina en los frutos, así como la aparición de nanoporos (Girard et al., 2012). Se ha propuesto la existencia de una familia de proteínas CUS-*like*/GDSL conservada en las plantas terrestres, y se han identificado ortólogos putativos en Arabidopsis y en el musgo *Physcomitrella patens* (Yeats et al., 2014).

Además de las proteínas **CUS**, se ha demostrado la participación de otra enzima que contribuye a la polimerización de la cutina, denominada BODYGUARD (**BDG**), una proteína de la familia α/β-hidrolasa, cuyo gen se expresa tanto en las células epidérmicas de las hojas, como en la endodermis de las raíces. El análisis de la mutante *bdg* de Arabidopsis, mostró que este gen es específico de la epidermis y su expresión muestra una ubicación extracelular, consistente con el papel sugerido como una enzima involucrada en la polimerización del monómero de cutina C_{18} (Kurdyukov et al., 2006b; Jakobson et al., 2016) (**Figura 4**).

Por otra parte, se ha propuesto un mecanismo alternativo para la síntesis de cutina, el cual no involucra enzimas, sino que se basa en el autoensamblaje de los monómeros de cutina, para formar nanoestructuras lipídicas de 40-200 nm de diámetro, que han sido denominadas **cutinsomas**. Se ha demostrado la participación de los **cutinsomas** en la biosíntesis de cutina, después de haberlos identificado en la epidermis de diferentes especies de plantas como tomate, Arabidopsis y *Ornithogalum umbellatum* (Domínguez et al., 2010; Segado et al., 2020). Los **cutinsomas** han sido observados mayoritariamente en la epidermis y en la cutícula naciente de frutos inmaduros de tomate, por medio de técnicas de inmunolocalización y por microscopía electrónica de transmisión, **TEM** (del inglés, *Transmission Electron Microscopy*) (Domínguez et al., 2010; Segado et al., 2020), aunque también en plantas de Arabidopsis (Stępiński et al., 2017; Stępiński et al., 2020) y de *O. umbellatum* (Stępiński et al., 2016). Asimismo, se ha demostrado bajo condiciones *in vitro*, que los **cutinsomas** crean un ambiente fisicoquímico que facilita la polimerización de monómeros de cutina sin participación de enzimas, formando de manera espontánea una película amorfa parecida a una cutícula (Heredia-Guerrero et al., 2008; Domínguez et al., 2010). En la **Figura 5**, se muestra un modelo propuesto del papel de estas estructuras en el ensamblaje de la cutina, comparado con el mecanismo que involucra la participación de enzimas.

Se ha sugerido la participación de constituyentes de la pared celular, como la pectina, que además de proporcionar soporte mecánico, podría jugar un papel activo en la formación de **cutinsomas**, uniéndose a monómeros de cutina. Guzman-Puyol y colaboradores (2015), observaron que durante la interacción pectina-análogos de monómeros de cutina, bajo condiciones *in vitro*, en una etapa inicial y en una solución a pH básico, algunas moléculas lipídicas se encuentran libres o, la mayoría, formando micelas. A medida que el medio se acidifica, se genera una proporción importante entre los grupos funcionales de ácido carboxílico (–COOH) y carboxilato ($–COO^-$) que presentan tanto los ácidos grasos, como la pectina; mientras que solo la pectina, forma mayoritariamente agregados de $–COO^-$, generando condiciones de pH neutro. Las cuales, favorecen la formación de nanopartículas mediante reacciones químicas de esterificación, que presentan núcleos lipídicos y sus capas están compuestas por la mezcla entre lípidos y pectina.

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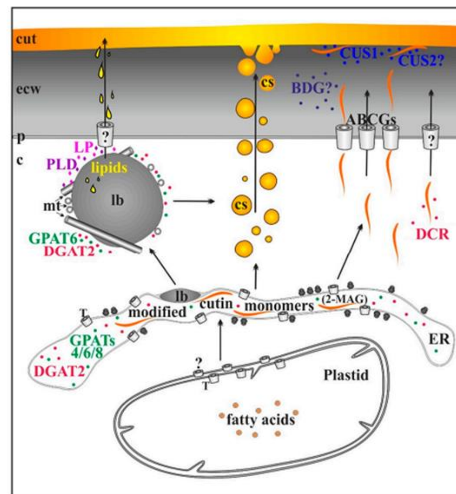

Figura 5. Modelo que ejemplifica los dos tipos de mecanismos para la biosíntesis de cutina. Mecanismo que involucra la participación de enzimas y mecanismo de autoensamblaje que involucra a los cutinsomas. Enzimas y transportadores BDG, CUS, DCR, DGAT2 (diacilglicerol aciltransferasa), GPAT y ABCG. **cs**, cutinsomas;. **cut**, cutícula; **ecw**, pared celular externa; **p**, membrana plasmática; **c**, citoplasma; **ER**, retículo endoplásmico; **lb**, cuerpos lipídicos; **LP**, lipasa; **PLD**, fosfolipasa; **mt**, microtúbulos. Imagen adaptada de Stępiński et al., (2020).

Por lo anterior, se ha propuesto que la agregación de los **cutinsomas** en la parte externa de la pared celular de las células epidérmicas, podría ser el primer paso en la formación de una cutícula temprana (procutícula). Es decir, durante las primeras etapas del desarrollo de las plantas, los **cutinsomas** participan en la formación de procutícula, mientras que en etapas posteriores, la adición de monómeros de cutina es impulsada por el mecanismo enzimático con la participación de **CUS1** y de **BDG**. Ambos mecanismos pueden actuar en secuencia y contribuir a la formación de la cutícula en etapas consecutivas del desarrollo de los órganos, como se propone para el fruto del tomate (Segado et al., 2020; Stępiński et al., 2020). Sin embargo, la contribución de los **cutinsomas** en la formación de la procutícula y la cutícula, necesita seguir investigándose.

I.1.2.2 Biosíntesis y polimerización de ceras.

En los últimos 40 años, la caracterización de mutantes deficientes en cera como *eceriferum* (*cer*, "sin cera") en Arabidopsis (Jenks et al., 1995; Jenks et al., 1996; Joubès y Domergue, 2018), *glossy* (*gl*) en maíz (*Zea mays*) (Bianchi et al., 1979), *bloomless* (*bm*) en sorgo (*Sorghum bicolor*) (Jenks et al., 1992) y *wax crystal-sparse leaf* (*wsl*) en arroz (*Oryza sativa*) (Yu et al., 2008), han permitido la identificación de genes implicados en la

biosíntesis de ceras cuticulares. Las ceras cuticulares consisten en una mezcla de compuestos alifáticos de cadena muy larga, que pueden contener de 22 a 48 átomos de carbono y se producen en el **RE** (Joubès y Domergue, 2018). Su biosíntesis depende del complejo enzimático ácido graso elongasa, **FAE** (del inglés, *Fatty acid elongase*) que está integrado por cuatro enzimas: KCS (del inglés, β-*ketoacyl-CoA synthase*), KCR (del inglés, β-*ketoacyl-CoA reductase*), una deshidratasa y una ECR (del inglés, *enoyl-CoA reductase*); el cual tiene como objetivo agregar carbonos para producir VLC-acil-CoA, los cuales pueden transformarse en ácidos grasos **VLCFA** libres y/o procesarse a través de dos caminos distintos. Uno de ellos es la vía de formación de alcoholes, el otro es la vía de formación de alcanos, dando lugar a la producción de toda la variedad de compuestos alifáticos que constituyen las ceras (Samuels et al., 2008; Haslam y Kunst, 2013) (**ver Figura 6**).

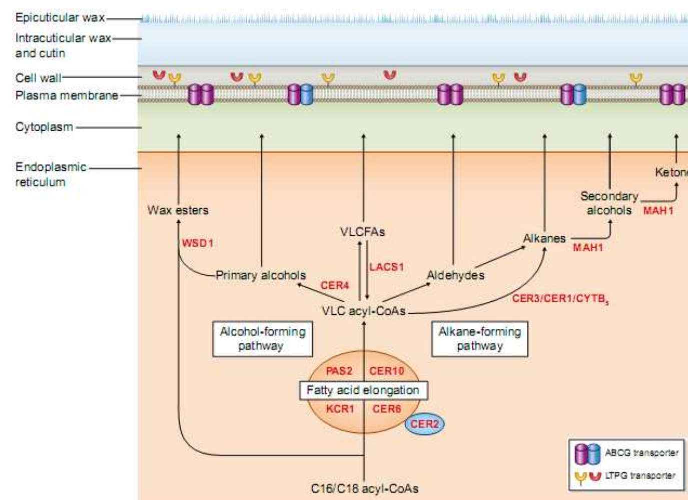

Figura 6. Modelo simplificado que ilustra los dos principales mecanismos para la biosíntesis de las ceras cuticulares, la vía de formación de alcoholes y la vía de formación de alcanos. La biosíntesis de las ceras cuticulares se inicia con la elongación de los ácidos grasos acil-coenzima A (CoA) C_{16} o C_{18} por el complejo elongasa de ácidos grasos y la proteína CER2. Posteriormente, los ácidos grasos de cadena larga (VLC-acyl-CoA) son modificados hacia aldehídos, alcanos, alcoholes secundarios y cetonas a través de la vía de formación de alcanos; o en alcoholes primarios y ésteres de cera, mediante la vía de formación de alcoholes. Los nombres en color rojo, indican las enzimas que se han identificado y se les ha asignado una función. Imagen adaptada de Lewandowska et al., (2020).

La **vía de formación de alcoholes**, también llamada de reducción, produce alcoholes primarios y ésteres de cera de número impar de átomos de carbono. En esta vía, se ha identificado la participación de CER4/FAR3, ya que su mutante de pérdida de función (*cer4*), muestra una reducción severa de los alcoholes primarios y ésteres de cera, por lo que la proteína codificada por este gen podría tener actividad ácido graso reductasa,

FAR (del inglés, *Fatty acyl-CoA reductase*) y ser la responsable de la síntesis de alcoholes en la epidermis de los tejidos aéreos y en las raíces (Rowland et al., 2006). Otra de las enzimas identificadas involucradas en esta vía, es la sintasa de ceras **WSD1** (del inglés, *Wax ester synthase* 1), que a través de la caracterización de la mutante *wsd1*, se reconoció como responsable de catalizar la formación de ésteres de cera mediante el uso de acil-CoA y alcoholes primarios como precursores (Li et al., 2008) (**Figura 6**). En Arabidopsis, los alcoholes primarios, tienen una longitud de cadena de C_{26} a C_{30} y representan del 12 al 14 % del total de las ceras; mientras que los ésteres, son componentes menores (< 5 %) y presentan una longitud de cadena que va desde C_{38} a C_{48} (Samuels et al., 2008; Joubès y Domergue, 2018).

Por su parte la **vía de formación de alcanos**, también llamada vía de descarbonilación, tiene como objetivo producir alcanos con número impar de C, aldehídos, alcoholes secundarios y cetonas, para constituir a las ceras en la mayoría de las plantas (Bernard y Joubès, 2013). Varias mutantes *cer* de Arabidopsis se han caracterizado bioquímicamente y se ha observado reducción tanto en el contenido de alcanos, como de las otras clases de compuestos que constituyen a las ceras en sus cutículas de hojas y de tallos, lo que indica su probable participación en la síntesis, regulación o transporte de precursores de los mismos (Jenks et al., 1995; Joubès y Domergue, 2018).

Las mutantes identificadas hasta ahora con un fenotipo claro en la síntesis de alcanos son *cer1/cer22* y *cer3* (Bernard y Joubès, 2013). De hecho, la mutante *cer1*, se caracteriza por una disminución significativa de alcanos, así como una nula producción de alcoholes secundarios y cetonas, acompañada de un ligero aumento en la cantidad de aldehídos; mientras que la mutante *cer3*, muestra una reducción significativa en el contenido de aldehídos, alcanos, alcoholes secundarios y cetonas. A partir de estos fenotipos, se propuso que *CER3/WAX2/YRE/FLP1/CER21* podría codificar para una potencial VLCFA reductasa, que cataliza la reducción de VLCFA a aldehídos y que *CER1/CER22*, podría codificar para una enzima formadora de alcanos (aldehído descarbonilasa), catalizando la descarbonilación de aldehídos a alcanos (Chen et al., 2003; Kurata et al., 2003; Bourdenx et al., 2011; Sakuradani et al., 2013). La prueba de que CER1 y CER3 actúan

como un complejo enzimático que cataliza la síntesis de VLC-alcanos fue evidenciada por la co-expresión de las dos proteínas en levadura (Bernard et al., 2012).

En plantas de Arabidopsis se presentan diferencias en los constituyentes de las ceras cuticulares de diferentes órganos. Mientras que los principales componentes en las hojas son los alcanos, en las ceras cuticulares del tallo, son los alcoholes secundarios y cetonas; y en las raíces los alcoholes primarios (Lee y Suh, 2015; Bernard y Joubès, 2013). Lo cual llevó a la hipótesis que los alcanos pueden sufrir más modificaciones en esta vía, como su oxidación secuencial para producir alcoholes secundarios y cetonas. En la búsqueda de genes que codifiquen para proteínas involucradas en la oxidación de lípidos relacionados con las ceras cuticulares, se identificó un gen que codifica para un citocromo P450, denominado **CYP96A15**/**MAH1**, como candidato para catalizar la formación de alcoholes secundarios y cetonas (**Figura 6**). El análisis del contenido de ceras en mutantes de este gen (*mah1-1*/*2*/*3*), indicó una severa reducción en ambos compuestos acompañado de un incremento en el contenido de alcanos (Greer et al., 2007).

En contraste a la biosíntesis, poco se conoce sobre la manera en que las ceras son secretadas y/o transportadas, desde el RE hacia la membrana plasmática y a través de los polisacáridos de la pared celular. Algunas son independientes del aparato de Golgi, como el transportador tipo ABC y las proteínas de transferencia de lípidos (**LTP**) (Samuels et al., 2008; Bernard y Joubès, 2013), mientras que otras, están mediadas por vesículas que transitan dentro de la red de Golgi y entre la interfaz RE-Golgi (McFarlane et al., 2014). El primer transportador ABC identificado para las ceras, fue **CER5**/**ABCG12**, gracias a la caracterización de la mutante *cer5*, la cual muestra un fenotipo de reducción del 50 % en el contenido de ceras en la cutícula de sus tallos y 15 % en la de sus hojas (Kunst y Samuels, 2009). La búsqueda de genes que codifican para proteínas ABC con un patrón de expresión similar a ABCG12, reveló que el transportador **ABCG11** es un candidato para la exportación de ceras (Bird et al., 2007). La mutante *abcg11* y la doble mutante *abcg11-abcg12* muestran una composición similar de ceras, sugiriendo que ambos transportadores actúan en la misma vía. Además, *abcg11* muestra fusión de órganos, defectos en la cutícula, mayor permeabilidad y contenido reducido de cutina;

lo que indica que ABCG11 también está involucrado en la exportación de monómeros de cutina (Bird et al., 2007; Panikashvili et al., 2007).

Basado en el análisis del transcriptoma de células epidérmicas de tallos de Arabidopsis, se identificaron siete genes que codifican para transportadores del tipo **LTP**, altamente expresados durante la formación de la cutícula, los cuales podrían desempeñar un papel en el transporte de precursores de ceras (Suh et al., 2005). Lo anterior, dio pie para el escrutinio y caracterización de las mutantes *ltpg1* y *ltpg2,* que están afectadas en los genes que codifican para glicosil-fosfatidil-inositol anclados a las LTP (**LTPG**); las cuales muestran un fenotipo de reducida acumulación de alcanos en la cutícula de sus tallos. Sin embargo, los cambios sutiles en la composición de las ceras observados en las mutantes *ltpg1* y *ltpg2*, han dado lugar a la propuesta de que se requieren múltiples LTP, con funciones redundantes, para exportar toda la diversidad de compuestos de la cutícula (Debono et al., 2009) (**Figura 6**).

I.1.3 Función de la cutícula en factores abióticos.

La cutícula es una estructura que gracias a sus propiedades físicas, químicas y morfológicas, presenta numerosas funciones de importancia fundamental para las plantas. Una de las mayores exigencias del estilo de vida terrestre de las plantas superiores, es mantener el equilibrio entre la pérdida de agua por transpiración, cuyo control se da por medio de los estomas o de la cutícula, y la absorción de agua desde la raíz; por ello, cualquier desequilibrio comprometerá la viabilidad y, por tanto, la adaptación de la planta (Riederer, 2006; Yeats y Rose, 2013). Algunos estudios han indicado que las ceras intracuticulares impiden, principalmente, la pérdida de agua no estomática (Zeisler-Diehl et al., 2018; Bueno et al., 2019); aunque también se ha destacado el papel de las ceras epicuticulares, lo cual se da de manera especie-específica (Jetter y Riederer, 2015; Bhanot et al., 2021). Lo anterior correlaciona con estudios que demostraron la expresión de genes implicados en la biosíntesis de ceras cuticulares, en respuesta a condiciones de sequía, en Arabidopsis, arroz y en pepino (Singh et al., 2018; Lokesh et al., 2019).

Además de la función señalada, y a pesar de restringir la difusión de agua, la cutícula es permeable a varias moléculas, una propiedad que es de valor agronómico, ya que permite la aplicación por aspersión de varios compuestos, como herbicidas o fertilizantes (Riederer y Schreiber, 2001; Nawrath, 2006). Asimismo, la cutícula contribuye a limitar la pérdida y absorción de gases como el CO_2, oxígeno, contaminantes inorgánicos del aire y compuestos orgánicos volátiles, como los terpenos. Además, esta interfaz planta-atmósfera, protege a la planta de las radiaciones de luz UV (Riederer, 2006), y establece límites físicos, cumpliendo una función mecánica que previene fusión de órganos al principio del desarrollo de la planta (Nawrath et al., 2013).

I.1.4 Función de la cutícula en interacciones bióticas.

Se ha reconocido desde hace mucho tiempo el importante papel de la cutícula vegetal como la primera barrera con la que se enfrenta un fitopatógeno, y que funciona tanto pasiva como activamente. Varios estudios han proporcionado evidencias que sugieren que los componentes cuticulares pueden actuar como un reservorio de señales para desencadenar las respuestas de defensa de las plantas, e incluso enviar señales que pueden ser utilizadas por los patógenos para iniciar sus procesos de crecimiento y desarrollo, y de esa forma lograr la infección (Nawrath et al., 2013; Serrano et al., 2014).

I.1.4.1 Interacción cutícula y bacterias patógenas.

Diversos estudios han revelado que la supervivencia e infección de patógenos bacterianos en la superficie de las plantas, se ven afectados por la integridad de la cutícula vegetal, principalmente por los componentes de las ceras, los cuales podrían influir en la resistencia o susceptibilidad a las infecciones (Aragón et al., 2017; Ziv et al., 2018). En la cutícula, los patógenos bacterianos suelen producir expolisacáridos y formar agregados que les ayuda a resistir las condiciones adversas y lograr la infección. Se sabe que la bacteria patógena *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000), produce el biosurfactante siringafactina, un compuesto que tiene propiedades tensoactivas que aumenta la permeabilidad de la cutícula de Arabidopsis y le facilitan a la bacteria su motilidad, permitiendo su migración hacia la zona donde hay mayor disponibilidad de nutrientes (Pfeilmeier et al., 2016). Por ejemplo, la mutante *sma4* de

Arabidopsis que presenta una cutícula permeable, con pérdida de función del gen *SMA4*, que codifica para la enzima LACS2 involucrada en la síntesis de la cutícula, mostró mayor susceptibilidad a *Pst* DC3000, pero mayor resistencia al hongo *B. cinerea* (Tang et al., 2007). Además de esta enzima, se han identificado factores de transcripción de la familia MYB, involucrados en la regulación de la síntesis de cutícula, y participan en la resistencia o susceptibilidad hacia *Pst* DC3000. Entre ellos está MYB30, el cual activa la expresión de genes biosintéticos de ceras, como *FATB*, *KCS1*, *KCS2*/*DAISY*, *KCS6* y *CER3,* resultando en la acumulación de cera foliar y en el contenido de ácido salicílico (Raffaele et al., 2009); así como en el desarrollo de resistencia hacia *Pst* DC3000 en las plantas que sobre-expresan el gen *MYB96* (Seo y Park, 2010).

Además, se ha observado que la acumulación de ciertos componentes de ceras, como aquellos que son sintetizados por la participación de CER1, tiene un efecto durante la interacción planta-bacteria. Plantas de Arabidopsis que sobre-expresan el gen *CER1* (*CER1ox*), promovieron la síntesis de alcanos de cadena larga y fueron más susceptibles a *Pst DC3000* (Bourdenx et al., 2011). Con estos estudios, se demuestra que la sola acumulación de ceras en la cutícula foliar no necesariamente contribuye a la resistencia hacia patógenos bacterianos, sino que va a depender del tipo de constituyente de las ceras epicuticulares.

I.1.4.2 Efecto de las ceras en el desarrollo de hongos patógenos.

La cutícula no solo funciona como barrera física entre las plantas y su entorno, sino también como fuente de moléculas de señalización para los patógenos, principalmente fúngicos, y la planta huésped. Está bien establecido que algunos componentes de las ceras epicuticulares en diferentes especies de plantas, como los aldehídos, alcoholes primarios de cadena larga y alcanos, son importantes en los procesos previos a la invasión de los hongos, como la adherencia, germinación de esporas y la formación de apresorios (Podila et al., 1993; Gniwotta et al., 2005; Inada y Savory, 2011; Nawrath et al., 2013). Por ejemplo, se ha demostrado que los aldehídos de cadena muy larga desencadenan la germinación de las esporas y la diferenciación del apresorio de *Blumeria graminis* f.sp. *hordei* (Hansjakob et al., 2010; Hansjakob et al., 2012). De igual forma, al silenciar el gen que codifica para el activador transcripcional de genes

biosintéticos de ceras en trigo *TaWIN1*, se redujo el contenido de aldehídos de cadena larga y se afectó negativamente la germinación de *B. graminis*. Sin embargo, al recubrir las hojas de estas plantas con ceras constituidas por aldehídos, se restauró la germinación de las esporas (Kong y Chang, 2018). Además de los aldehídos de cadena larga, se ha identificado la participación de los alcoholes primarios en el inicio de la infección de los hongos fitopatógenos, *Puccinia graminis* f.sp. *tritici* y de *Colletotrichum gloeosporioides*, los cuales dependen de la presencia de alcoholes primarios de cadena muy larga C_{24} en la cutícula de sus plantas hospederas, trigo (*Triticum aestivum*) y aguacate (*Persea americana*), respectivamente (Podila et al., 1993).

Gniwotta y colaboradores (2005), demostraron que la germinación de *Erysiphe pisi* era mayor en el lado adaxial comparada con el abaxial de las hojas de los guisantes, lo cual se relacionó con una alta concentración de alcoholes primarios de cadena larga C_{26}. Otro estudio, demostró que el alto contenido de alcoholes primarios y de aldehídos en la cutícula de las hojas de la mutante *cer1* de Arabidopsis y en el tallo de la mutante *cer3,* inhibieron los procesos de pre-invasión, germinación y formación de apresorio del hongo biótrofo *Golovinomyces orontii* (Jenks et al., 1995; Inada y Savory, 2011). Curiosamente, la sobreexpresión del gen *CER1* en Arabidopsis, promovió la biosíntesis de alcanos de cadena larga, resultando en mayor susceptibilidad a la infección por el hongo necrótrofo *Sclerotinia sclerotiorum*, destacando que los alcanos de cera también desempeñan funciones de señalización importantes (Bourdenx et al., 2011). Estos componentes también afectan negativamente la formación de estructuras de pre-infección, lo cual se observó al caracterizar el fenotipo de la mutante *irg1* de *Medicago truncatula*. La mutación en el gen *PALM1*/*IRG1*, originó resistencia hacia los hongos *Phakopsora pachyrhizi*, *Puccinia emaculata* y *Colletotrichum trifolii,* relacionado con la acumulación de alcanos C_{29} y C_{31} y la reducción en el contenido de alcoholes primarios en las hojas (Uppalapati et al., 2012).

Además de los componentes de ceras, también se ha identificado la participación de los monómeros de cutina como reguladores de las relaciones bióticas. Durante las primeras etapas de la infección, las cutinasas fúngicas secretadas actúan sobre la cutícula liberando monómeros de cutina, y a medida que los hongos los perciben, se

incrementa la actividad de la enzima requerida para la penetración. Por ejemplo, los monómeros de cutina inducen la germinación y formación de órganos de infección especializados como el apresorio del hongo *Magnaporthe grisea* (Gilbert et al., 1996) y el tubo germinativo de *Erysiphe graminis* (Francis et al., 1996). Los monómeros de cutina también inducen la actividad de la proteína cinasa, **LIPK** (del inglés, *Lipid-induced Protein Kinase*) en *C. trifolii*, esencial para la formación de estructuras de infección del hongo (Dickman et al., 2003).

En resumen, los estudios descritos dejan claro que la composición química, estructura y cantidad de ceras epicuticulares, así como de monómeros de cutina, son factores importantes que influyen en diversos procesos de desarrollo de los hongos patógenos, para el éxito de la infección de su hospedero.

I.1.4.3 Interacción mutantes afectadas en cutícula con hongos patógenos.

Los patógenos atacan a las plantas porque a lo largo de la evolución han adquirido la capacidad de utilizar y depender de las sustancias producidas por éstas, como acto de supervivencia. Muchas de estas sustancias están contenidas en los protoplastos de las células, por lo que los patógenos fúngicos han desarrollado diversos mecanismos para acceder a ellas, que van desde la entrada a través de las aberturas naturales, hasta aquellos que les permitan atravesar las barreras externas, como la cutícula y la pared celular. Entre estos últimos, están la formación de estructuras especializadas de infección, como en el caso del hongo hemibiótrofo *Magnaporthe oryzae* que requiere la formación del apresorio, para penetrar directamente la superficie de las hojas a través de la fuerza mecánica. Otro de los mecanismos involucra la secreción de enzimas degradadoras de la cutícula y de la pared celular, ejemplo de ello son los hongos necrótrofos, como *B. cinerea*, *Alternaria brassicicola* y *S. sclerotiorum,* que pueden detectar componentes cuticulares para secretar enzimas hidrolíticas, como las cutinasas y estimular los procesos de pre-penetración, como la germinación de las esporas y posterior formación de estructuras de infección, que le permiten ingresar a través de la cutícula, matando células epidérmicas (Göhre y Robatzek, 2008; Dodds y Rathjen, 2010; Mengiste, 2012). Lo anterior, se suma a las diversas evidencias que han mostrado el impacto de los defectos en la cutícula de las plantas en las diferentes etapas de la

infección por los hongos, y cómo estos defectos podrían conducir a la inmunidad o susceptibilidad de la planta ante el ataque de los patógenos (Chassot et al., 2008; Serrano et al., 2014; Aragón et al., 2017).

Uno de los primeros intentos para elucidar el efecto de los componentes de la cutícula, así como las alteraciones en su estructura, sobre la inmunidad hacia hongos patógenos, fue el estudio de Sieber y colaboradores (2000), quienes generaron plantas transgénicas de Arabidopsis denominadas plantas CUTE, que sobre-expresan una cutinasa fúngica y presentan fusión de órganos; además de mostrar inmunidad frente a *B. cinerea*, a pesar de presentar modificaciones en su cutícula, como mayor permeabilidad. Para fortalecer lo observado, se empezó con la caracterización de la interacción entre mutantes cuticulares y hongos fitopatógenos. Tal fue el caso con la mutante de Arabidopsis *lacs2*, deficiente en la enzima acil-CoA sintetasa 2 que participa en la vía de síntesis de la cutina y de ceras, la cual muestra una reducción cinco veces de la cantidad total de ácidos grasos ω-hidroxilados y sus derivados, comparados con la planta tipo silvestre. Además, estas modificaciones llevaron a una fuerte resistencia de la mutante *lacs2* a los hongos necrótrofos *B. cinerea* y *S. sclerotiorum*, mientras que en presencia de los hongos *Plectosphaerella cucumerina* (Bessire et al., 2007) y *A. brassicicola*, no se observó inmunidad (Tang et al., 2007).

De manera similar, otras mutantes de Arabidopsis como, *bdg*, *lcr*, *fdh*, *abcg32*/*pec1* y *gl1,* principalmente con defectos en diferentes pasos en la formación de cutina, presentaron una cutícula permeable y resistencia a *B. cinerea* (Bessire et al., 2007; Chassot et al., 2007; Tang et al., 2007; Voisin et al., 2009; Bessire et al., 2011). En la mayoría de estas mutantes, el grado de permeabilidad de la cutícula ha demostrado una relación directa con el grado de resistencia hacia *B. cinerea* (Voisin et al., 2009).

I.2 Mecanismos de defensa inducibles: Sistema inmune innato vegetal.

Durante su desarrollo, las plantas se enfrentan a una gran variedad de patógenos. Para considerarse patógeno, los microorganismos deben acceder al interior de la planta, ya sea penetrando la superficie de la hoja o la raíz directamente o entrando a través de aberturas naturales, como los estomas o bien mediante heridas. Debido a su naturaleza

sésil, las plantas no pueden evitar el peligro simplemente alejándose o escondiéndose, además, carecen de un sistema inmunitario adaptativo, como si lo tienen los animales. Las células vegetales son autónomas, desde el punto de vista para percibir o reconocer a los patógenos y activar mecanismos de defensas que los detengan, por lo que han desarrollado un sistema inmune innato, capaz de distinguir las señales exógenas derivadas de los patógenos, de las propias (endógenas) (Jones y Dangl, 2006).

El sistema inmune innato vegetal, está constituido por niveles de defensa sucesivos descritos en el modelo más aceptado hasta el momento, conocido como *zig-zag*, que involucra dos ramas de estrategias moleculares de defensa (**Figura 7**). En la primera, intervienen mecanismos de reconocimiento de patrones moleculares del patógeno (**PAMP**) o de patrones asociados al daño en la planta derivado por la acción del patógeno (**DAMP**) a través de los receptores de reconocimiento de patrones (**PRR**) anclados a la superficie celular, para inducir un conjunto de respuestas denominadas colectivamente como inmunidad desencadenada por estos patrones, **PTI** (Jones y Dangl, 2006; Boller y Felix, 2009). De manera paralela, los patógenos han co-evolucionado con su hospedero y desarrollaron estrategias de supresión de la **PTI**, mediante la síntesis de moléculas llamadas **efectores**, que le permiten asegurar su colonización. Sin embargo, para contrarrestar esta función, las plantas desarrollaron un segundo nivel de inmunidad innata activada por efectores denominada **ETI**, basada en el reconocimiento directo o indirecto de los efectores por parte de un grupo de receptores o proteínas de resistencia (**R**) (Dodds y Rathjen, 2010). La mayoría de los genes *R* codifican para receptores del tipo **NB-LRR** (con dominio de unión a nucleótidos y repeticiones ricas en leucinas), los cuales confieren resistencia a diversos hongos, bacterias, virus e insectos patógenos. **ETI** es una respuesta **PTI** acelerada y amplificada en el sitio de infección, resultando en una respuesta celular hipersensible, que limita la expansión del patógeno al resto de las células.

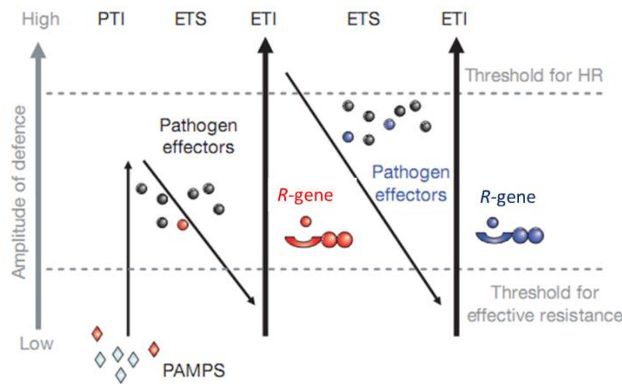

Figura 7. El modelo de *zig-zag* que describe la co-evolución de los mecanismos de defensa de las plantas y de los patógenos. El primer nivel de defensa de la planta es la inducción de PTI, tras la detección de PAMPs. Los patógenos pueden evadir la PTI o bloquear su activación produciendo efectores que atenúan la respuesta de defensa de susceptibilidad desencadenada por efectores (ETS). El reconocimiento de efectores induce una fuerte respuesta de defensa que conduce a la muerte celular (HR) y finalmente a ETI. Imagen adaptada de Jones y Dangl (2006).

La activación de las respuestas inmunes en las plantas, desencadena cascadas de eventos de señalización complejos; por lo tanto, la resistencia o susceptibilidad de una especie vegetal depende de la interacción entre el patógeno y la planta. La colonización por el patógeno y el desarrollo de la enfermedad, solo tiene lugar cuando el patógeno tiene la capacidad de evadir las barreras físicas de defensa y las respuestas inmunes **PTI** y **ETI**.

I.2.1 Alteración del flujo iónico y activación de MAPK.

Tras el reconocimiento de los PAMP/DAMP o de efectores, se desencadena una serie de respuestas tempranas que incluyen: flujo de iones a través de la membrana plasmática, que provoca la entrada de H^+ y de Ca^{2+} en la célula, y la salida de K y aniones al espacio extracelular. La concentración de Ca^{2+} es de particular interés, ya que se sabe que éste funciona como segundo mensajero en varios procesos celulares. La aplicación exógena de PAMP como flagelina (flg22) y EF-Tu (elf8), de DAMP como Pep1 (del inglés, *Plant elicitor peptides*) y el efector AvrRpm1 de *P. syringae* DC3000 a plántulas de Arabidopsis, genera un aumento en el calcio citosólico, que está asociado con la activación transcripcional de algunos genes de defensa (Ranf et al., 2011; Flury et al., 2013). El flujo de calcio también es requerido para que se lleve a cabo la respuesta hipersensible y la producción de **ROS**. Además de los flujos de iones, se activan cascadas de fosforilación de gran importancia en distintas vías de transducción y amplificación de

señales intracelulares, mediadas por **MAPK** cinasas o por cinasas dependientes de calcio (**CDPK**), que fosforilan factores de transcripción implicados en la expresión de genes relacionados con la defensa (Boller y Felix, 2009).

I.2.2 Estrés oxidativo.

Otra de las respuestas tempranas que ocurren es el denominado estrés oxidativo que involucra la producción de **ROS**. Las plantas están expuestas a un ambiente oxidante y producen constantemente **ROS** en los cloroplastos, mitocondrias, peroxisomas y otros sitios de la célula como el apoplasto, como consecuencia de procesos metabólicos primarios como la fotosíntesis y la respiración. La diversidad de **ROS**, incluyen el radical superóxido (O_2^-), el peróxido de hidrógeno (H_2O_2), el radical hidroxilo (OH^-), el singlete de oxígeno ($^1O_2$) y el óxido nítrico (NO) (O'brien et al., 2012; Lehmann et al., 2015). Sin embargo, tras el reconocimiento del patógeno, las células vegetales producen principalmente H_2O_2, localizado en el apoplasto, cuya producción resulta de la participación de enzimas productoras de **ROS**, como las NADPH oxidasas, localizadas en la membrana plasmática (RBOH), peroxidasas y poliaminas oxidasas (PAO) (Torres et al., 2006). Las NADPH oxidasas de plantas son similares a las RBO (del inglés, *Respiratory Burst Oxidase*) de mamíferos y se identificaron por primera vez en arroz. En Arabidopsis, se han identificado 10 genes *Atrboh* (del inglés, *respiratory burts oxidase homologs*), que codifican para NADPH oxidasas, pero solo *AtrbohD y AtrbohF* se han asociado con funciones redundantes en la defensa contra los patógenos *Hyaloperonospora arabidopsidis* y *P. syringae* (Torres y Dangl, 2005; Torres et al., 2006). Debido a su alta toxicidad, las ROS han estado implicadas no sólo en funciones antimicrobianas directas, sino también en mecanismos de señalización celular para la activación de otros mecanismos de defensa; por ejemplo, la producción de fitoalexinas, el reforzamiento de la pared celular, la respuesta hipersensible y la activación de genes de defensa.

I.2.3 Respuesta hipersensible y muerte celular programada.

La muerte celular programada es un mecanismo imprescindible en todos los organismos multicelulares, desde regular procesos de crecimiento y desarrollo, hasta responder a condiciones ambientales. También, juega un papel importante en la inmunidad vegetal, activándose durante la **ETI** y dando lugar a la respuesta

hipersensible, la cual está precedida por eventos tempranos anteriormente descritos. Se ha demostrado que la **HR** es altamente efectiva para el control de la infección causada por patógenos hemibiótrofos (*Xantomonas campestris* y *P. syringae*) y biótrofos (*H. arabidopsidis*), ya que limita la disponibilidad de nutrientes en el sitio de la infección, con la finalidad de delimitar su propagación (Boller y Felix, 2009). Sin embargo, en patógenos necrótrofos como *B. cinerea* y *S. sclerotiorum*, son los propios patógenos los que inducen la **HR** produciendo enzimas degradadoras de la pared celular, para macerar el tejido y beneficiar su propagación en la planta (Govrin y Levine, 2000).

I.2.4 Implicación de las fitohormonas en la defensa.

Las respuestas de defensa son el resultado de una compleja interacción entre diversas rutas, entre ellas la producción de **ROS**, de óxido nítrico, **HR** y producción de fitohormonas. Dentro de las principales hormonas implicadas en la interacción planta-patógeno se ha identificado al SA, al JA y al ET. Sin embargo, también se ha descrito la participación de otras hormonas como el ABA, las auxinas, las citoquininas (CK), las giberelinas (GA) y los brasinoesteroides (BR) (**Figura 8**). La caracterización genética y la contribución de estas rutas en los procesos de desarrollo y de defensa en las plantas, se ha dado a partir de la identificación de mutantes alteradas en su biosíntesis, percepción y transducción de señales. Dependiendo de la interacción planta-patógeno, se llegan a activar una o varias rutas de defensa mediadas por estas hormonas, las cuales pueden interactuar de manera sinérgica o antagónica, formando una compleja red de intercomunicación, que proporciona a la planta capacidad para ajustar sus respuestas de defensa (Pieterse et al., 2009; Pieterse et al., 2012; Denancé et al., 2013). Estas moléculas están involucradas en lo que parecen ser dos vías principales de señalización para la defensa de patógenos: una vía dependiente de ácido salicílico y una vía independiente de SA que involucra a JA y ET. La señalización por SA regula positivamente la defensa de la planta típicamente (pero no exclusivamente), contra patógenos biótrofos (aquellos que requieren tejido vegetal vivo para asegurar su supervivencia), mientras que las vías JA/ET comúnmente se han descrito como necesarias para la resistencia a patógenos necrótrofos (aquellos que completan su ciclo de vida usando tejido vegetal muerto) e insectos herbívoros (Denancé et al., 2013) (**Figura 8**).

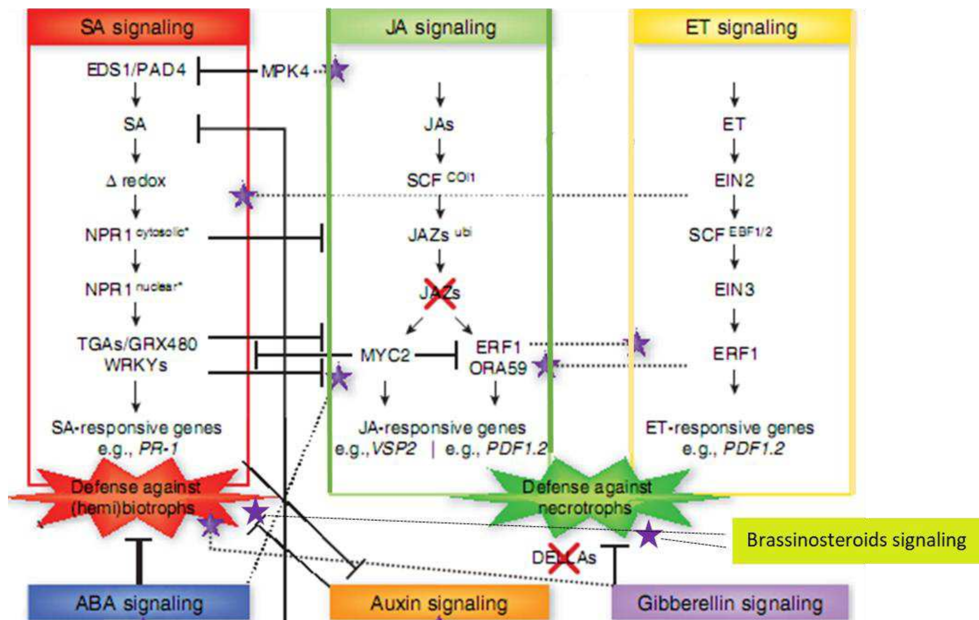

Figura 8. Redes de comunicación entre las fitohormonas en la respuesta inmune de las plantas. La comunicación entre las vías de señalización hormonal proporciona a la planta una gran capacidad reguladora que puede adaptar su respuesta de defensa a diferentes tipos de patógenos. Los componentes de transducción de señales con efecto positivo o de activación son mostrados con estrellas púrpuras; el efecto inhibitorio es mostrado con líneas truncadas (⊥). Imagen adaptada de Pieterse*,* et al. (2009).

El papel del SA durante las interacciones planta-patógeno, fue registrado por primera vez en plantas de tabaco y pepino en 1990. Las plantas con mayor resistencia al virus del mosaico del tabaco (TMV) mostraron una fuerte acumulación de esta molécula, mientras que en plantas susceptibles a TMV, los niveles de SA se redujeron significativamente (Malamy et al., 1990). Diversos estudios han mostrado que los niveles endógenos de SA aumentan en el tejido infectado y que la aplicación exógena de esta molécula incrementa la resistencia a muchos patógenos (Pieterse et al., 2012; Maruri-López et al., 2019). Dentro de la ruta de señalización mediada por SA, destaca el gen *NPR1* (del inglés, *Non-expressor of PR genes* 1) por su papel como regulador maestro positivo de esta vía, actuando como activador transcripcional de genes relacionados con la defensa. La mayoría de éstos se activan transcripcionalmente y son llamados genes ***PR*** (del inglés, *Pathogenesis-Related*), entre ellos destacan *PR1* y *PR5*, los cuales son utilizados como marcadores de esta ruta de señalización. Asimismo, se conocen varios factores de transcripción del tipo WRKY que desempeñan importantes funciones rio abajo de *NPR1*, tales como mediar las respuestas de defensa en las plantas (Kunkel y Brooks, 2002; Pieterse et al., 2012).

La señalización dependiente de JA y de ET, se produce a través del incremento de su síntesis en respuesta a heridas, insectos y patógenos necrótrofos. Mutantes de Arabidopsis afectadas en la producción y en la percepción de JA, como la triple mutante *fad3-fad7-fad8* y la mutante *jar1* (del inglés, *jasmonic acid resistant 1*), respectivamente, exhiben una marcada susceptibilidad a patógenos necrótrofos, como son los hongos *A. brassicicola*, *B. cinerea*, *P. cucumerina* y a la bacteria *Pectobacterium carotovora*. En Arabidopsis, la señalización por JA se ha demostrado que se lleva a cabo a través de dos rutas mediadas principalmente por factores de transcripción, una de ellas incluye a MYC2/JIN1 (del inglés, *Jasmonate insensitive 1*) y la otra, a varios miembros de la familia AP2/ERF (del inglés, *Apetala2/Ethylene-Response Factor*). La ruta MYC2 regula positivamente la expresión de genes marcadores sensibles a JA inducibles por heridas e insectos, como *VSP2* (del inglés, *Vegetative Storage Protein* 2) y *LOX2* (del inglés, *Lipoxygenase 2*), cuando la síntesis de ET está ausente. Mientras que la ruta por ERF, se induce sinérgicamente en presencia de ET, entre ellos destacan los factores de transcripción sensibles a JA/ET, incluidos ERF1 (del inglés, *Ethylene-Response Factor* 1) y ORA59 (del inglés, *Octadecanoid-Responsive Arabidopsis* 59), los cuales regulan positivamente la expresión de genes que responden a JA/ET, como *PDF1.2* (del inglés, *Plant Defensin* 1.2) (Pieterse et al., 2012). Al igual que en la vía por JA, en la vía del ET, los ERF son los principales reguladores en respuesta a patógenos, destacando EIN2 y EIN3 que activan las respuestas de defensa. ET participa con las vías de SA y de JA, ya sea de forma antagónica o induciéndolas para lograr respuestas de defensa personalizadas.

En los últimos años, se ha descrito la participación de nuevos mediadores de las respuestas de defensa, como son el ácido abscísico y los brasinoesteroides. El ácido abscísico, además de controlar procesos de desarrollo de la planta (germinación, dormancia, respuesta a sequía, apertura de estomas), puede funcionar como un regulador positivo o negativo en función del tipo de interacción planta-patógeno. Mutantes de sobreexpresión con alteración en la síntesis o en la señalización de ABA, en tomate (*sitiens*) y en Arabidopsis (*abi1-1*, *abi2-1*, *aba1-6*, *aba2*-12, *aao3-2* y *pyr1-pyl1-pyl2-pyl4*), conducen a una mayor resistencia a diferentes patógenos, como son *B. cinerea*, *P. syringae*, *F. oxysporum*, *P. cucumerina* y *Hyaloperonospora parasítica*

(Denancé et al., 2013). Por su parte, el papel de los BA se comenzó a estudiar por la implicación del receptor citoplásmico BAK1 (del inglés, *Bri1-Associated receptor Kinase*) en la respuesta inmune PTI, principalmente en la regulación de la muerte celular y por interactuar físicamente con varios receptores del tipo PRR. Asimismo, se agregó a la lista de los componentes compartidos por las vías de los BA y en la inmunidad por PTI, la participación del receptor BIK1 (del inglés, *Botrytis-Induced Kinase 1*).

I.3 Estrategias de infección de *Botrytis cinerea*.

B. cinerea es un importante hongo patógeno de plantas muy común en la naturaleza, ubicado como el segundo patógeno de importancia agrícola, después de *Magnaporthe oryzae*, debido a su amplio espectro de especies hospederas (Dean et al., 2012). Se considera un necrótrofo típico, ya que provoca la muerte celular programada en el hospedero para lograr la infección. La disponibilidad de la secuencia del genoma y una variedad de herramientas moleculares (facilidad de transformación para obtener mutantes *knockout* o para lograr silenciamiento de genes), junto con su relevancia económica, han contribuido a que *B. cinerea* sea el patógeno fúngico más estudiado (Van Kan, 2006). Este hongo es más destructivo en tejidos maduros o senescentes de plantas dicotiledóneas y puede permanecer de manera quiescente durante un tiempo considerable antes de la pudrición de los tejidos, cuando la fisiología del huésped cambia y el ambiente es poco propicio, puede ocurrir una infección desde la etapa de plántula hasta la maduración del fruto. En la **Figura 9**, se esquematiza el proceso de infección de *B. cinerea*: las esporas, una vez sobre la hoja deben germinar y formar estructuras de infección como el apresorio, para atravesar la superficie foliar cuya capa más superficial, es la cutícula. A diferencia de los apresorios formados en especies de *Colletotrichum* o de *Magnaporthe*, los apresorios producidos por *B. cinerea* carecen de una pared que lo selle. Esto significa, que mientras las especies mencionadas ingresan al interior de las células ejerciendo presión física solamente a través de la generación de grandes cantidades de presión osmótica, *B. cinerea* no tiene esta capacidad. Para compensarla, *B. cinerea* produce y libera, a través del apresorio, enzimas líticas como cutinasas y lipasas, así como fitotoxinas (Abuqamar et al., 2006; Van Kan, 2006).

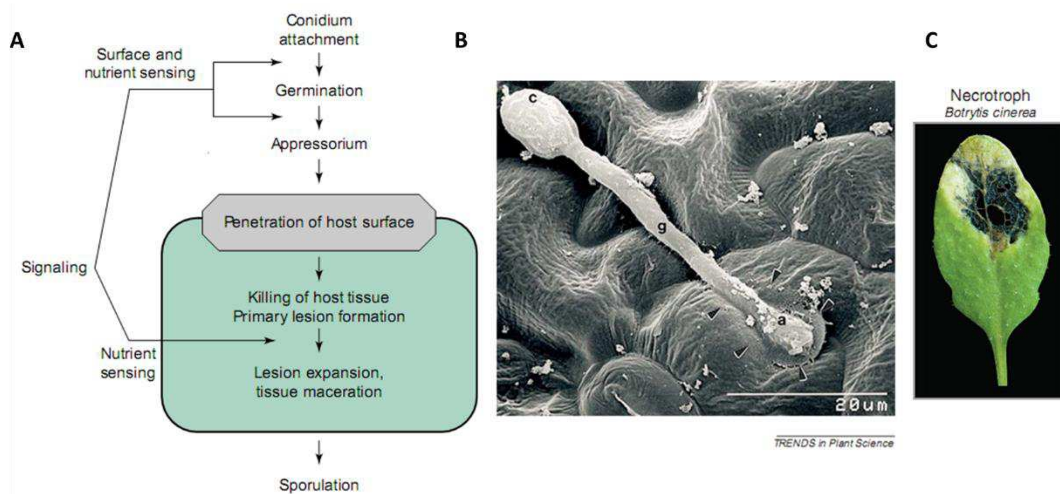

Figura 9. Procesos de infección de *B. cinerea* en plantas. **A)** Etapas de desarrollo de *B. cinerea* que ocurren durante la infección. **B)** Germinación y desarrollo del apresorio en la superficie de una hoja de tomate. Abreviaturas: **a**, apresorio; **c**, conidio; **g**, tubo germinativo. **C)** Síntoma de enfermedad en hojas de Arabidopsis causada por *B. cinerea*. Imagen adaptada de Van Kan (2006).

B. cinerea atraviesa las células epidérmicas secretando enzimas degradadoras de la pared celular **CWDE** (del inglés, *Cell Wall Degrading Enzymes*), como pectinasas, celulasas y hemicelulasas. En consecuencia, las plantas que actúan como hospederos comunes o susceptibles a esta especie, acumulan **ROS** en la membrana plasmática, para desencadenar un estrés oxidativo, lo que lleva a la muerte celular programada (Abuqamar et al., 2006). Mientras que, en hospederos no comunes o no compatibles, el sistema inmune vegetal se inhibe mediante la producción de pequeñas moléculas de ARN, **sRNA** (del inglés, *small RNAs*) por parte de *B. cinerea* que tienen como finalidad silenciar los genes de defensa de la planta, mediante el mecanismo de RNA de interferencia (Weiberg et al., 2013). El objetivo final de un patógeno vegetal necrótrofo no es matar su planta hospedera *per se*, sino descomponer la biomasa vegetal y utilizarla para su desarrollo. Una característica común de todas las especies de plantas colonizadas por *B. cinerea*, es su alto contenido de pectina en la pared celular. Especies de plantas con bajo contenido de pectina se consideran hospederos pobres para *B. cinerea*. Por lo tanto, se ha postulado que la preferencia de hospedero de *B. cinerea* refleja la posesión de una maquinaria pectinolítica (Govrin y Levine, 2000; Van Kan, 2006).

2. Preguntas de investigación e Hipótesis

2.1 Preguntas de investigación

- ¿Cómo influyen los cambios en el contenido de cutina y de ceras de la cutícula de Arabidopsis, en la interacción con *B. cinerea*?
- ¿Cambios en el contenido de ceras, tienen el mismo efecto de inmunidad que los cambios en el contenido de cutina en la interacción Arabidopsis-*B. cinerea*?
- ¿El aumento de permeabilidad cuticular, junto con la producción constitutiva de ROS, presentes en las mutantes afectadas en el contenido de cutina y en ceras, son los principales mecanismos que participan en la resistencia a *B. cinerea*?
- ¿Qué mecanismos moleculares involucrados en las respuestas de defensa hacia *B. cinerea*, se desencadenan en las mutantes afectadas en la estructura cuticular?

2.2 Hipótesis

La modificación en la estructura cuticular en Arabidopsis, derivada de los componentes principales, cutina y ceras, asociada con el incremento de la permeabilidad y aumento en los niveles de producción de ROS tiene una correlación directa hacia la resistencia a *B. cinerea*.

3. Objetivos.

3.1 Objetivo general.

Identificar y caracterizar elementos moleculares implicados en las respuestas de defensa a *Botrytis cinerea*, en las mutantes afectadas en la composición de la cutícula de *Arabidopsis thaliana*.

3.2 Objetivos particulares.

- Caracterizar la permeabilidad de la cutícula foliar y la producción de ROS de las mutantes afectadas en la composición de los componentes de la cutícula.
- Establecer la posible relación entre la composición y estructura de la cutícula y su efecto en la inmunidad hacia *B. cinerea*.
- Caracterizar el transcriptoma en ausencia y en presencia del fitopatógeno *B. cinerea* en las mutantes afectadas en: cutina (*bdg* y *lacs2-3*), ceras (*cer3-6* y *cer1-4*) y en ambos componentes (*eca2*).

CAPÍTULO II.
Artículo de revisión.

En este capítulo se presenta un artículo de revisión que se publicó en el primer año de doctorado. Fue parte de una sección de la edición especial: **The plant cuticle: old challenges, new perspectives**, en la revista *Journal of Experimental Botany*; el cual permitió responder una de las primeras preguntas de investigación de este trabajo de tesis, a través de la revisión y síntesis de la literatura relevante y reciente acerca de cómo los componentes de la cutícula vegetal tienen algún efecto sobre los microorganismos fitopatógenos y microorganismos benéficos localizados en la filósfera.

The intimate talk between plants and microorganisms at the leaf surface.

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REVIEW PAPER

The intimate talk between plants and microorganisms at the leaf surface

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Abstract

The plant epidermis or cuticle is constantly exposed to external and internal environmental factors, including an enriched and diverse community of bacteria, yeast, fungi, viruses, and mites. It is not only where the plant has its first physical barrier, but also where organisms can be recognized and potentially where the plant defense responses can be triggered. The plant cuticle is a polymeric composite formed by an array of structurally and chemically heterogeneous compounds, including cutin and wax. A few studies have shown that cuticular components are essential and important drivers of the structure and size of the bacterial community. On the other hand, cuticular components are also important for both pathogens and plants, to initiate the pre-invasion and infection process and to activate the innate immune response, respectively. In this review, we explore current knowledge on the role of the cuticle during the intimate interactions between plants and microorganisms, in particular pathogenic and non-pathogenic bacteria and fungi. Finally, we propose new perspectives on the potential use of this information for agriculture.

Key words: Bacteria, cutin monomers, epicuticular waxes, fungi, innate immunity, microorganism, plant cuticle, phyllosphere.

Introduction

Besides bark, the cuticle is the structure that covers the surface of the epidermal cells of the aerial parts of higher plants, such as petals, seeds, fruits and leaf structures, including stomata and the trichomes that arise from the epidermis and extend outward (Riederer and Schreiber, 2001; Müller and Riederer, 2005; Riederer, 2007; Jones *et al.*, 2014; Fich *et al.*, 2016). As a physical barrier, one of the main functions of the cuticle is to prevent water loss and at the same time avoid the penetration of solutes and pollutants into the plant, and to protect it against irradiation, xenobiotics, herbivores, and microorganisms (Müller and Riederer, 2005; Domínguez *et al.*, 2011; Nawrath *et al.*, 2013). The cuticle is considered to be a composite formed by an array of structurally and chemically heterogeneous compounds, dominated by the biopolyester cutin, which consists of a matrix of mid-chain hydroxy and/or epoxy C_{16} and/or C_{18} fatty acid monomers (Domínguez *et al.*, 2011; Nawrath *et al.*, 2013; Fernández *et al.*, 2016). It also has waxes, which are a complex mixture of compounds containing predominantly very-long-chain fatty acids (VLCFAs; C_{20}–C_{40}), alcohols, alkanes, aldehydes, esters, and triterpenoids, which can be found within the cutin matrix (intracuticular waxes) or on its surface (epicuticular

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waxes). Epicuticular waxes accumulate as an amorphous layer or in the form of discontinuous crystals, which gives them a glaucous appearance (Gniwotta *et al.*, 2005; Jeffree, 2007; Jetter *et al.*, 2007; Domínguez *et al.*, 2011; Yeats and Rose, 2013; Fernández *et al.*, 2016). Other minor compounds present in the cuticle are phenolics, cutan, polysaccharides, and mineral elements. Previous reviews have described how the heterogeneous structural and chemical nature of the cuticle varies between species, genotypes, organs, and developmental stages (Müller and Riederer, 2005; Jetter *et al.*, 2007; Yeats and Rose, 2013; Fernández *et al.*, 2016; Vacher *et al.*, 2016).

The plant epidermis is constantly exposed to external and internal environmental factors, including an enriched and diverse community of bacteria, yeast, fungi, viruses, and mites. Such complexity can be easily found to average approximately 10^6–10^7 cells cm^{-2} of leaf (Lindow and Brandl, 2003; Baldwin *et al.*, 2017). For this reason, besides its central function as a protective barrier, the cuticle plays an important role during the interaction with leaf microbiota, including pathogenic and non-pathogenic microorganisms (Vorholt, 2012; Vacher *et al.*, 2016). In order to avoid the invasion of pathogenic microorganisms, plants have evolved inducible defense responses. The initial defense responses constitute the so-called plant innate immunity, which is triggered by recognition of the chemical molecules microbe-, pathogen- and/or damage-associated molecular patterns (MAMPs, PAMPs and/or DAMPs, respectively), which are released during the interaction with microbes and recognized by pattern recognition receptors (PRRs) (Boller and Felix, 2009). Plant immunity can be divided into two categories, PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Fig. 1A) (Zipfel, 2014; Conrath *et al.*, 2015). PTI includes a broad spectrum of defenses such as accumulation of reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK)-dependent signaling cascades, induction of defense genes and synthesis of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Boller and Felix, 2009). However, pathogens often produce effectors that interfere with the activation of PTI and are recognized by specific resistance (R) proteins, inducing the ETI that triggers a hypersensitive response (Jones and Dangl, 2006; Craig *et al.*, 2009). Both mechanisms work coordinately to block the invasion of pathogens, locally and systemically by priming the defense responses (Craig *et al.*, 2009; Tsuda and Somssich, 2015). The priming defense includes the systemic and induced acquired resistance (SAR and IAR) that depend on the coordinated effect of the plant hormones SA, JA, and ET (Conrath *et al.*, 2015). Remarkably, fragments of the cuticle have been described as being perceived as DAMPs by the plant and by the microorganism, activating different process during pathogenesis (Reina-Pinto and Yephremov, 2009).

Here we describe recent evidence of how differences in the chemical and physical composition of the cuticle regulate the intimate interactions between the plants and pathogenic and non-pathogenic microorganisms, putting special emphasis on bacteria and fungi. Additionally, we describe how plant defense responses are modulated by the cuticular-derived DAMPs. Changes in the plant–microbe interactions due to cuticular modifications are listed in Table 1. Finally, we discuss how this information can be used to improve plant breeding and biological control programs.

Bacterial communities associated with the cuticular surface

The plant leaf surface is considered to be a harsh environment for microorganisms, since they are exposed to adverse conditions such as supra-optimal radiation, limited moisture, precipitation, and dramatic shifts in temperature (Hirano and Upper, 2000; Marcell and Beattie, 2002; Zinsou *et al.*, 2006). Nevertheless, the aerial parts of plants provide a heterogeneous environment, termed the phyllosphere, for a large number of microbial communities including bacteria, yeasts, viruses, and fungi (Lindow and Brandl, 2003; Whipps *et al.*, 2008). Several excellent reviews have summarized earlier work on phyllosphere microbiology and ecology (Beattie and Lindow, 1999; Leveau, 2007; Whipps *et al.*, 2008; Vorholt, 2012; Vacher *et al.*, 2016).

Bacteria are the most abundant colonizers of the phyllosphere and referred to as epiphytes. Beneficial bacteria predominate in this biotype, but some phytopathogens also take advantage of the conditions they find there and use it for the first stage of infection. Epiphytic bacteria are capable of thriving on plant leaf surfaces and it has been estimated that their global population could reach up to 10^{26} cells (Lindow and Brandl, 2003; Whipps *et al.*, 2008). Bacterial communities are generally dominated by the phylum *Proteobacteria*, including *Methylobacterium*, *Sphingomonas*, *Xanthomonas*, *Pantoea*, and *Pseudomonas* (Delmotte *et al.*, 2009; Vorholt, 2012; Vogel *et al.*, 2016), although other bacterial phyla such as *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* have also been identified at the core of the phyllosphere community (Vorholt, 2012; Bulgarelli *et al.*, 2013). Most of them colonize the plant surfaces as large and heterogeneous aggregates, which allows them to withstand the surrounding conditions (Kinkel and Lindow, 1993; Vorholt, 2012). These aggregate structures confer on them the advantage of maintaining a hydrated surface through production of extracellular polymeric substances (Lindow and Brandl, 2003; Whipps *et al.*, 2008). This production plays an important role (adhesion) in the epiphytic style of some of the pathogenic bacteria that colonize the plant surface. Furthermore, bacteria must also deal with leaf traits, including the hydrophobic composition of the cuticle and an irregular distribution of nutrients, due to variation in the availability of plant-leached metabolites at the leaf surface, including several carbon sources (i.e. amino acids, organic acids, sugar alcohols), and volatile organic compounds such as isoprenes, monoterpenes, and methanol (Hirano and Upper, 2000; Lindow and Brandl, 2003; Whipps *et al.*, 2008; Vacher *et al.*, 2016). These characteristics suggest that both epiphytic pathogenic and non-pathogenic bacterial populations are directly influenced by certain environmental conditions and indirectly by the metabolism of their host, so that only adapted bacteria can

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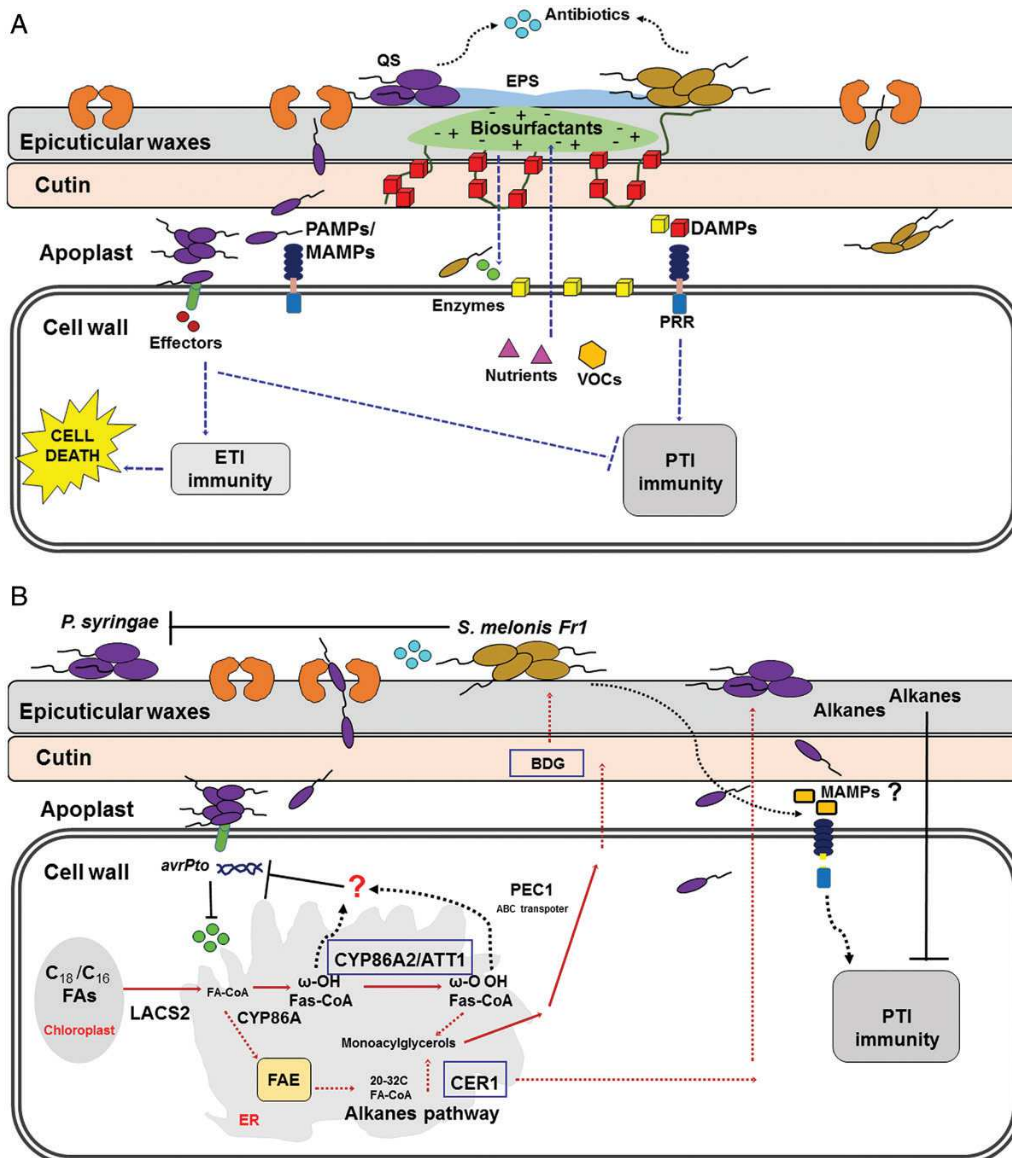

Fig. 1. Bacterial communities on the leaf surface. (A) In order to survive and thrive, epiphytic bacteria produce antibiotics and extracellular polymeric substances (EPS) that help them to form aggregates and induce quorum sensing (QS), which is biosurfactant production to increase cuticle permeability and make plant-leached metabolites (i.e. nutrients) and volatile organic compounds (VOCs) available to the leaf surface. Pathogenic bacteria are able to grow epiphytically on the surface and then change to an endophytic lifestyle. To avoid the plant cuticle, bacteria swim toward openings, the stomata (shown in orange); once they are in the apoplast plants can recognize the invasion by the interaction of PRRs with MAMPs/PAPMs triggering the PTI, as well as DAMPs (shown as yellow cubes for cell-wall degradation products and red cubes for cuticle monomers). Nevertheless, bacteria can produce effectors to inhibit PTI, but if these elicitors are recognized by the plant, then ETI is induced, leading to a cell-death response. (B) Changes in cutin structure modify epiphytic bacteria. For instance, PEC1 (an ABC transporter) and α/β hydrolase (BDG) involved in cutin biosynthesis are important to beneficial bacterial communities. Similarly, LACS2 shows an increase of the beneficial bacterium *Sphingomonas melonis* Fr1 (brown). In particular, an increase in *S. melonis* Fr1 induces a growth inhibition of the pathogen *Pseudomonas syringae* (purple), probably by the production of antibiotics (blue circles), nutrient competition and/or activation of PTI by recognition of MAMPs (yellow squares). Interestingly, the product of CYP86A2/ATT1 indirectly represses the *P. syringae* type III gene *avrPto* (green circles). This inhibition might be mediated by certain fatty acids related to biosynthesis of cutin monomers (ω-OH Fas-CoA or ω-OOH Fas-CoA). The cuticular wax enzyme CER1 appears to be important in fatty acid elongation (FAE) to produce alkanes as components of waxes acting as signals in resistance to *P. syringae*.

survive. Because of the hydrophobic nature of the cuticle, epiphytic bacteria are able to produce compounds with surfactant properties (Fig. 1A). An example is syringafactin, produced by the well-characterized pathogenic bacterium *Pseudomonas syringae*; it facilitates flagellum-dependent motility and increased cuticle permeability necessary for diffusion and solubilization of substrates to occur, making the substrates available at the leaf surface (Knoll and Schreiber, 1998; Burch *et al.*, 2012; Pfeilmeier *et al.*, 2016). Since they obtain nutrients from living plant cells, these pathogens are considered to be biotrophic (Melotto *et al.*, 2008; Baker *et al.*, 2010; Pfeilmeier *et al.*, 2016), but during its infection cycle, *P. syringae* is able to epiphytically colonize the plant surface at the cuticular level, before entry and growth into the apoplast (Fig. 1A; Hirano and Upper, 2000). It would be interesting to know why *P. syringae* chooses to be epiphytic

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Table 1. *Cuticular mutants with modified responses during the plant–microbe interactions*

Mutant	Plant	Gene name	Gene locus	Gene product	Function of gene product	Phenotype			Reference
						Cuticle permeability	Bacteria	Fungi	
att1/cyp86a2	Arabidopsis	*CYP86A2*	At4g00360	CYP86A2 Cytochrome P450 family	ω- Hydroxylase Biosynthesis of cutin	+	Susceptible to *P. syringae*	Resistant to *B. cinerea*	Tang *et al.* (2007), Xiao *et al.* (2004)
lacs2/sma4/ bre1	Arabidopsis	*LACS2*	At4g49430	LACS2 Long-chain acyl-CoA synthetase	Attachment of CoA to free fatty acids Biosynthesis of cutin	+	Susceptible to *P. syringae*	Resistant to *B. cinerea* and *S. clerotiorum*	Bessire *et al.* (2007), Schnurr *et al.* (2004), Tang *et al.* (2007)
lcr	Arabidopsis	*CYP86A8*	At2g45970	CYP86A8 Cytochrome P450 family	ω- Hydroxylase Biosynthesis of cutin	+	nt	Resistant to *B. cinerea.*	(Bessire *et al.* (2007), Wellesen *et al.* (2001)
fdh	Arabidopsis	*FDH*	At2g26250	β-Ketoacyl-CoA synthase (KCS)	VLCFA biosynthesis	+	nt	Resistant to *B. cinerea*	Voisin *et al.* (2009), Yephremov *et al.* (1999)
cer1	Arabidopsis	*CER1*	At4g02205	CER1 VLC-aldehyde decarbonylase putative	Formation of VLC alkane Biosynthesis of waxes	+	Susceptible to *P. syringae*	Resistant to *B. cinerea* and *S. sclerotiorum*	Aarts *et al.* (1995), Bourdenx *et al.* (2011)
cer3/wax2	Arabidopsis	*CER3/WAX2*	At5g57800	CER3 VLC-Acyl-CoA reductase putative	Catalysing the synthesis of VLC-alkanes Biosynthesis of waxes	+	nt	Disruption of *G. orontii* prepenetration process	Inada and Savory (2011), Samuels *et al.* (2008)
bdg/cb5	Arabidopsis	*BDG*	At1g64670	A cuticle-related α/β hydrolase domain protein	Unknown function in Biosynthesis of cutin	+	nt	Resistant to *B. cinerea*	Chassot *et al.* (2007), Jakobson *et al.* (2016), Kurdyukov *et al.* (2006*a*)
pec1	Arabidopsis	*PEC1*	At2g26910	ATP binding cassette 32 (ABC32) transporter	Formation of the cuticular layer by exporting cutin precursors	+	nt	Resistant to *B. cinerea*	Bessire *et al.* (2011)
hth/ace	Arabidopsis	*HTH*	At1g72970	Long-chain FA ω-alcohol dehydrogenase	Oxidation of long-chain ω-hydroxy FAs to ω-oxo FAs	±	nt	± Resistant to *B. cinerea*	Bessire *et al.* (2007), Kurdyukov *et al.* (2006*b*)
myb96	Arabidopsis	*MYB96*	At5g62470	MYB96 ABA-responsive R2R3-type Transcription factor	Transcriptional activator of genes encoding VLCFAs involved in cuticular wax biosynthesis	+	nt	Resistant to *B. cinerea*	Benikhlef *et al.* (2013), Seo *et al.* (2011)
aba2 and *aba3*	Arabidopsis	*ABA2* *ABA3*	At1g52340 At1g16540	Short-chain alcohol dehydrogenase. Mo-cofactor sulfurase.	Alcohol dehydrogenase (NAD) and Mo cofactor sulfurase activity ABA Biosynthesis	+	nt	Resistant to *B. cinerea*	L'Haridon *et al.* (2011)

Table 1. *Continued*

Mutant	Plant	Gene name	Gene locus	Gene product	Function of gene product	Phenotype			Reference
						Cuticle permeability	Bacteria	Fungi	
gl11	*Z. mays*	*GL11*	Its locus has been mapped to chromosome 2	*Gl11* gene product remain to be elucidated	Cuticular wax biosynthesis	nt	nt	Disruption of *B. graminis* prepenetration process	Hansjakob *et al.* (2011)
irg1/palm1	*M. truncatula*	*IRG1/PALM1*	MTR_5g014400	Cys_2His_2 zinc finger transcription factor, PALM1	Formation of epicuticular wax crystals	nt	nt	Disruption of *P. pachyrhizi*, *P. emaculata*, and *C. trifolii* prepenetration process	Uppalapati *et al.* (2012)
cyp83a1	Arabidopsis	*CYP83A1*	At4g13770	CYP83A1 The cytochrome P450 monooxygenase	Biosynthesis of glucosinolates	nt	nt	Delayed germination and differentiation of appressoria of *E. cruciferarum*	Weis *et al.* (2014)

nt, not tested.

before infecting the internal tissues of the plant through natural openings. Several authors have identified possible evolutionary forces that may explain why foliar pathogenic bacteria multiply on plant surface. Some of them respond to a cell population density, mainly by quorum sensing (Hirano and Upper, 2000; Melotto *et al.*, 2008; Pfeilmeier *et al.*, 2016). Once a certain size of bacterial population is reached, foliar pathogenic bacteria are ready to enter the apoplast (Fig. 1A). Significant advances have been made in understanding how these bacteria use several strategies to deal with plant innate immunity. However, it is not completely known if specific components of the leaf cuticle play a direct role in early communication between plants and in the formation of epiphytic plant bacterial colonies.

Below, we describe the probable roles that the plant cuticle structure plays for both epiphytic pathogenic and non-pathogenic bacteria populations living at the phyllosphere.

Changes in cuticle modify plant–pathogenic bacteria interactions

Cuticular mutants have been evaluated during the interaction between plants and *Pseudomonas syringae*. One of them, the *att1* Arabidopsis mutant derived from the gene *ATT1* (*Aberrant* induction of *Type Three* genes1) coding for a cytochrome P450 monoxygenase enzyme, CYP86A2, has been characterized as playing a role in the formation of C_{16} and C_{18} ω-hydroxy fatty acids, which are essential for cutin structure. *att1* has a cutin content reduced to 30% compared with the wild-type and shows enhanced susceptibility to the virulent *P. syringae* pv. *tomato* (*Pst*) DC3000 (Xiao *et al.*, 2004). Remarkably, CYP86A2 represses the gene expression of the bacterial type III protein avrPto, involved in *P. syringae* virulence (Fig. 1B; Xiao *et al.*, 2004). This suggests that CYP86A2 indirectly represses bacterial gene expression from the intercellular space, either by means of certain lipids such as cutin monomers or by other fatty acids related to cutin monomers that are synthetized by this enzyme (Fig. 1B; Xiao *et al.*, 2004; Reina-Pinto and Yephremov, 2009).

Another example was described by Tang *et al.* (2007), who identified the *sma4* mutant (*symptoms to multiple avr4*), which displayed severe disease symptoms after infection with *P. syringae* DC3000. The *SMA4* gene was identified by positional cloning as encoding LACS2, a member of the family of acyl-activating enzymes LACS (acyl-CoA synthetase), involved in cutin biosynthesis (Fig. 1B; Schnurr *et al.*, 2004). Additionally, Bourdenx *et al.* (2011) reported the characterization of the Arabidopsis *ECERIFERUM1* (*CER1*), predicted to encode the enzyme CER1. *cer1* is the only mutant described so far to be involved in alkane biosynthesis, with reduced wax content related to plant–bacteria interactions (Aarts *et al.*, 1995; Bourdenx *et al.*, 2011). Interestingly, C*ER1*-overexpressing lines, where the amount of alkane is increased and it accumulated on the leaf surface, show reduced leaf permeability and enhanced susceptibility to *P. syringae* DC3000 (Bourdenx *et al.*, 2011). VLCFA derivates have been reported as signaling molecules of the plant immune response to pathogen attacks (Shah, 2005), but their

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over-accumulation might lead to defense response inhibition (Bourdenx *et al.*, 2011).

Finally, it has been shown that some lipids can participate in the activation of SAR. Xia *et al.* (2009) found that an acyl carrier protein, ACP4, involved in the biosynthesis of cuticular C_{16} or C_{18} fatty acids, is required to perceive the mobile SAR signal in distal tissues of Arabidopsis. The *acp4* mutant was able to generate the mobile signal required for inducing SAR, but was unable to induce a systemic response, suggesting that loss of signal perception in *acp4* is related to defective cuticles (Xia *et al.*, 2009). From the above results we can conclude that there is evidence that cuticle-derived signals are involved in the early interactions between plants and pathogenic bacteria. However, further investigations are needed to clarify these observations.

For instance, analysis of the composition of leaf cutin monomers was not performed in the mentioned studies, with the exception of Xiao *et al.* (2004), where alteration of leaf cuticle in the *att1*/*cyp86a2* plants was observed using transmission electron microscopy images. Additionally, answering the following questions, might improve our understanding about cuticular components acting as signaling molecules during plant–bacteria interactions: What are the cuticle related-molecules that induce bacterial pathogenesis? And why do certain cuticle elements allow or limit pathogenic bacterial colonization? Identification of these cuticular components, for example using quantitative analytical techniques, during the interaction with bacteria will bring us useful information to develop new strategies for plant protection.

Cuticular composition influences colonization of epiphytic non-pathogenic bacteria

Only a few studies have been focused on finding out if a specific cuticular trait, such as wax or cutin composition, exerts an effect on colonization of non-pathogenic bacterial. To cite some examples, cuticular mutants of maize and Arabidopsis have been studied for this purpose (Marcell and Beattie, 2002; Reisberg *et al.*, 2013; Bodenhausen *et al.*, 2014; Ritpitakphong *et al.*, 2016). Here we describe in detail these reports.

Marcell and Beattie (2002) used four maize (*Zea mays* L.) glossy mutants, *gl1*, *gl3*, *gl4* and *gl5*/*gl20*, which have altered composition of their juvenile waxes and a glossy appearance of the leaves. These mutants were previously characterized to possess a similar wax composition on their juvenile leaves, compared with adult wild-type leaves (Marcell and Beattie, 2002). The composition of the adult waxes of the wild-type maize leaves consisted of smaller percentages of primary alcohols (14%) and aldehydes (9%), and higher percentages of esters (42%) and alkanes (17%) compared with the composition of the wild-type juvenile leaf waxes. Besides these features, it is important to note that *gl1*, *gl3*, *gl4*, and *gl5*/*gl20* mutants produced less epicuticular crystalline waxes, showing a more hydrophilic surface compared with the wild-type (Marcell and Beattie, 2002). All these properties were used to study the influence of the leaf surface waxes during plant–bacteria interaction and thereby to evaluate the colonization of two bacterial species, the common saprophyte *Pantoea agglomerans* and the pathogen *Clavibacter michiganensis*. *gl1*, *gl5*/*gl20*, and *gl3* retained bigger bacterial populations (approximately 10^6 cells per gram of leaf) than the wild-type 96 h post-inoculation, while the *gl4* mutant showed a clear difference in the dynamics of *C. michiganensis* and *P. agglomerans*, reaching a population density of 2×10^7 cells per gram of leaf after the same period of time. The authors also evaluated the effect of the density of crystal waxes of the wild-type leaves on bacterial colonization, introducing gaps among them. These surface-altered leaves supported larger bacterial populations of *P. agglomerans* compared with wild-type non-altered leaves. According to these results, it is worthy of note that changes in the composition of the cuticular waxes can be an important factor in determining the size of a specific bacterial population, whether it is a beneficial or a pathogenic microorganism.

Reisberg *et al.* (2013) used the Arabidopsis *eceriferum* mutants (*cer1*, *cer6*, *cer9*, and *cer16*) involved in cuticle biosynthesis to evaluate whether these mutations would affect the bacterial populations from natural colonizing phyllosphere bacteria (Reisberg *et al.*, 2013). The authors revealed that members of the bacterial genera *Sphingomonas*, *Pseudomonas*, *Rhodococcus*, *Methylobacterium*, and *Burkholderiales*, known to be phyllosphere colonizers in other plant species, are part of the Arabidopsis 'core' community (Delmotte *et al.*, 2009; Vorholt, 2012). However, other phylotypes such as *Pedobacter* and *Flavobacterium* were identified and seemed to be more specific to Arabidopsis (Vorholt, 2012; Bodenhausen *et al.*, 2013). Additionally, Bodenhausen *et al.* (2014) studied the changes induced in the well-defined bacterial communities, previously identified, on the leaf surface of Arabidopsis mutant impaired in cutin biosynthesis *lacs2* and *pec1* (Fig. 1B) (Delmotte *et al.*, 2009; Vorholt, 2012; Bodenhausen *et al.*, 2013; Bodenhausen *et al.*, 2014). From these analyses the authors determined that cuticle synthesis is an important factor for the bacterial community composition, since more members of genus *Variovorax* were observed on *lacs2* and *pec1*, but fewer bacteria of the genus *Rhodococcus*, *Sphingomonas* sp. *Fr1*, and *S. phyllosphaerae*, compared with wild-type plants (Fig. 1B).

By analysing the phyllosphere of cuticular mutants *bdg* and *lacs2* (Fig. 1B), it was determined that microbes on the plant surface are important factors for plant resistance against the fungal pathogen *Botrytis cinerea* (Ritpitakphong *et al.*, 2016). Under non-sterile conditions *bdg* and *lacs2* are fully resistant to the necrotrophic *B. cinerea* (Serrano *et al.*, 2014). However, when the plants were grown and inoculated under sterile conditions, the mutant *bdg* became as susceptible as the wild-type plant, while the *lacs2* mutant retained resistance (Ritpitakphong *et al.*, 2016). Remarkably, *bdg* resistance was restored when *lacs2*-phyllosphere microbiome was applied on the leaf surface. The most abundant bacterial genera identified in the cuticular mutants were *Pseudomonas* and *Rhizobium*, while in the wild-type plants *Burkholderia* was the most abundant genus. Interestingly, a *Pseudomonas* sp., isolated from the microbiome of *bdg*, provided resistance to *B. cinerea* on wild-type plants (Ritpitakphong *et al.*, 2016). These results indicated that the microbiome of the leaf

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surface of Arabidopsis cuticular mutants can protect against the fungal pathogen *B. cinerea*.

Summarizing the above observations, the model plants maize and Arabidopsis have provided clear supporting evidence that epicuticular wax and cutin composition have an impact on diversity and abundance of non-pathogenic bacterial communities. However, many questions await answers. It will be necessary to determine if there is a direct effect of a specific wax composition on how cutin compounds might influence the beneficial bacterial colonization and how these traits lead and shape a specific target bacterial genus.

Regulation of plant–fungi interaction by the cuticle

Plants and fungi have coexisted and coevolved during the last 400–460 million years. They developed complex, beneficial and harmful interactions during this time (Heckman *et al.*, 2001). One example of this complexity is the mutually beneficial symbiotic interactions with arbuscular mycorrhiza (Bonfante and Genre, 2008) and the success of this interaction is clear, since approximately 80% of plant species are associated with a symbiotic fungus (Karandashov *et al.*, 2004). On the other hand, diseases caused by fungi are one of the most economically important factors to affect agriculture (Dean *et al.*, 2012). For instance, in the USA alone the cost of the annual control of fungus-induced diseases can reach $23.5 billion (Rossman, 2009). In the aerial parts of the plant, the first contact between plants and fungi takes place at the cuticle and for this reason, as expected, this structure plays an important regulatory role during these interactions. Here we describe how changes in plant cuticle structure and chemistry regulate the pre-penetration and colonization process of fungi and induction of the plant defense response.

How can cuticular wax and cutin modify the development of fungal pre-penetration structures?

Plant–fungi intimate interactions are triggered by the ability of the microorganisms to attach to the leaf surface. Epicuticular and intracuticular waxes make the plant cuticle a highly hydrophobic interface, repelling water, aqueous solutions, polar mucilages, and microorganism with polar coatings (Müller and Riederer, 2005; Nawrath *et al.*, 2013). In some cases, in order to overcome the cuticle barrier, fungal spores excrete extracellular material containing hydrophobic proteins that function as adhesive compounds to the leaf surface (Carver *et al.*, 1999). For the powdery mildew *Blumeria graminis*, almost immediately after the first contact takes place at the epicuticular wax layer, production of a proteinaceous matrix outside the conidia is induced and coordinates the germination and the differentiation of the conidia (Nielsen *et al.*, 2000; Wright *et al.*, 2002). Recently, using a forward genetics approach in *Medicago truncatula Tnt1* insertion lines, the *inhibitor of rust germ tube differentiation1* (*irg1*/*palm1*) mutant was identified, turning out to be a transcription factor, *PALM1*, which is implicated in the induction of genes involved in epicuticular wax biosynthesis (Uppalapati *et al.*, 2012). Additionally, the mutant showed reduced amounts of abaxial epicuticular wax crystals and reduced leaf surface hydrophobicity, inhibiting the pre-infection process of two rust pathogens, *Phakopsora pachyrhizi* and *Puccinia emaculata*, and the anthracnose pathogen *Colletotrichum trifolii* (Uppalapati *et al.*, 2012). However, not only the physical properties of cuticle, namely hydrophobicity, determine the beginning of the pre-penetration process, but also the cuticle's chemical composition.

Previously, it has been described that chemical components of cuticular wax, including long-chain alcohols, triterpenoids, and aldehydes, are important during the initial fungal pre-invasion stages, such as spore germination and appressorium formation (Ahmed *et al.*, 2003; Inada and Savory, 2011). Interestingly, the pre-penetration process of the biotrophic fungus *Golovinomyces orontii*, which causes powdery mildew disease on Arabidopsis, was inhibited in the *eceriferum* (*cer*) mutants *cer3*/*wax2* but not in *cer1* (Inada and Savory, 2011). CER1 (Fig. 1B) and CER3/WAX2 have been described as participating in wax biosynthesis (Chen *et al.*, 2003; Rowland *et al.*, 2007; Bourdenx *et al.*, 2011; Mao *et al.*, 2012), and additionally, CER1 interacts with itself and with CER3 and CYTB5s to reconstitute biosynthesis of a very-long-chain alkane in a heterologous yeast system (Bernard *et al.*, 2012). Interestingly, CER1 and CER3/WAX2 are similar in amino acid sequence but have different cuticular properties (Aarts *et al.*, 1995; Chen *et al.*, 2003; Bourdenx *et al.*, 2011). *cer1* and *cer3*/*wax2* have been described as possessing a reduced amount of cuticular wax on both stems and leaves (Jenks *et al.*, 1995), but only *cer3*/*wax2* showed increased cuticle permeability (Inada and Savory, 2011). This last observation might suggest that pre-invasion inhibition is due to changes in permeability. However, a similar fungal development stage is not affected in another Arabidopsis mutant with high permeability (*lacs2*; Bessire *et al.*, 2007). This indicates, once more, that this phenotype can be due to changes in the cuticular wax chemical composition rather than changes in cuticle permeability. For instance, the lack of epicuticular waxes on the barley mutant *cer* did not affect the pre-penetration process, but removal of the major wax components hexacosanol and hexacosanal reduced conidial germination and differentiation of *Blumeria graminis* by 20% (Zabka *et al.*, 2008). Remarkably, cuticular wax components, the very-long-chain aldehydes, have been described as inducing the pre-penetration process of *B. graminis* in barley and maize (Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011; Weidenbach *et al.*, 2015), and have been characterized as stimulating the migration of the nucleus inside the conidia, close to the site of primary germ-tube emergence (Hansjakob *et al.*, 2012). It is worth mentioning that this chemical regulation by the plant seems to be very specific between the host and its fungal invader; for example, only the cuticular terpenoids from avocado can modify the pre-penetration development of its own pathogen, *Colletotrichum gloeosporioides*, but they did not affect the growth of other *Colletotrichum* species (Podila *et al.*, 1993).

Besides cuticular waxes, cutin monomers can also regulate the initial events during plant interactions with fungi. Several

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reports have shown that perception of C_{16} cutin monomers activates the fungal pre-penetration machinery, including the induction of cuticle-degrading enzymes such as cutinase (Li *et al.*, 2002; Ahmed *et al.*, 2003). In fact, cutinases have been described as being required for spore attachment on the plant surface of several phytopathogens including *Uromyces viciae-fabae*, *Colletotrichum graminicola* and *Blumeria graminis* (Deising *et al.*, 1992; Pascholati *et al.*, 1992, 1993). Interestingly, activation of these enzymes is also important for the subsequent stages of pathogenesis. For instance, the expression of the *cutinase2* gene (*CUT2*) from *Magnaporthe grisea* is up-regulated during appressorium development and fungal penetration (Skamnioti and Gurr, 2007). Remarkably, *cut2* mutants are less pathogenic in rice and barley, due to changes in the development of pre-penetration structures, such as germ tubes and appressoria. However, those defects can be fully restored by exogenous application of synthetic cutin monomers (Skamnioti and Gurr, 2007). It has been suggested that cutin monomers can also modify the interaction between fungi and the plant root. Even if roots do not contain cutin, but only the structurally related compound suberin, it has been recently reported that arbuscular mycorrhizal fungi can perceive cutin monomers as a signal (Wang *et al.*, 2012). This observation raises an interesting open question: could the cutin monomers have modulated the interactions between ancient land plants (without roots) and fungi resembling mycorrhizas since the beginning of plant–fungi interactions (Murray *et al.*, 2013)? This highlights the importance of cutin monomers during the early steps of plant–fungi interactions and its characterization could unravel the molecular mechanisms that both organisms use to communicate with each other.

How can the cuticle affect the development of penetration structures?

Once a pathogens has attached to the leaf surface and pre-penetration structures are developed, the infection of the fungus into the plant intracellular space can begin. Several strategies are used by fungi to penetrate the plant cuticle, including entry through natural openings such as stomata and inflicted wounds and/or use of osmotic force and lytic enzymes. However, other pathogens such as *Magnaporthe oryzae*, *Ustilago maydis* and *Colletotrichum* sp., use the specialized penetration structures called appressoria (Zeilinger *et al.*, 2016). Appressorial development is initiated by sensing the cutin monomer 16-hydroxy hexadecanoic acid by the putative sensor proteins Msb2 and Sho1, which activate an MAPK-dependent signaling pathway leading to a genome-wide transcriptional reprograming in the fungus (Lanver *et al.*, 2014; Ryder and Talbot, 2015). Multiple reports have described an intimate regulation by cuticular wax and cutin monomers of appressorial development in different fungal species. For example, changes in the metabolic enzymes involved in the biosynthesis of VLCFAs such as CYP96B22 and CYP83A1 and the wax mutant *glossy11* showed a modification of appressorial development in *Magnaporthe oryzae*, *Erysiphe cruciferarum*, and *Blumeria graminis* in barley, Arabidopsis, and maize, respectively (Hansjakob *et al.*, 2011; Delventhal *et al.*, 2014; Weis *et al.*, 2014). Interestingly, formation of appressoria in the mutants *cyp83a1* and *glossy11* can be chemically restored by exogenous application of the C_{26} aldehyde *n*-hexacosanal (Hansjakob *et al.*, 2011; Weis *et al.*, 2014). Additionally, cutin monomers can also modify appressorium development of *Blumeria graminis* and *Ustilago maydis* (Francis *et al.*, 1996; Mendoza-Mendoza *et al.*, 2009). Nevertheless, not all fungi use appressoria to infect plants, and other pathogens such as *B. cinerea* use lytic enzymes to degrade the cuticle. Additionally, it has been previously described that germination of *B. cinerea* spores in the presence of waxes leads to changes in gene expression and conidia development (Leroch *et al.*, 2013). Remarkably, in a follow-up characterization, the authors described Msb2 as a sensor of the cuticle chemical components that regulate germling and hyphae development, leading to the formation of the infection structure by activation of a *BMP1* MAPK-dependent signaling pathway in *B. cinerea* (Leroch *et al.*, 2015). These results point out a similar regulation, induced by cuticular wax and cutin monomers, of several pathogenic fungi with different penetration strategies.

Plant defense responses against fungi induced by cuticular components

Changes in cuticular wax and cutin play an important role during plant–pathogen interactions and have been extensively described in several reviews (Kachroo and Kachroo, 2009; Reina-Pinto and Yephremov, 2009; Serrano *et al.*, 2014). As a physical barrier, one of the cuticle's main functions is to regulate abiotic and biotic stresses. For this reason, changes in cuticle permeability lead to high sensibility to different abiotic stresses, such as drought and herbicide treatments, mainly because molecules can be released faster from the apoplast and/or can quickly penetrate into the plant cell (Nawrath *et al.*, 2013). Surprisingly, changes in the plant cuticle during interaction with the necrotrophic fungus *B. cinerea* lead to an unexpected outcome. Defense mechanisms against *B. cinerea* are enhanced in Arabidopsis and *Solanum lycopersicum* mutants with increased leaf permeability and modified cutin biosynthesis, leading to full resistance against this pathogen (Bessire *et al.*, 2007; Chassot *et al.*, 2007; Tang *et al.*, 2007; Li *et al.*, 2007; Voisin *et al.*, 2009; Nawrath *et al.*, 2013; Serrano *et al.*, 2014). In agreement with these observations, it has recently been described that overexpression of the negative regulator of wax biosynthesis, AP2/ERF-type transcription factor DEWAX, leads to an increase leaf permeability and ROS accumulation, up-regulation of defense-response genes and resistance to *B. cinerea* in Arabidopsis and *Camelina sativa* plants (Ju *et al.*, 2017). One hypothesis to explain this response is that once *B. cinerea* spores have landed on the more permeable leaf surface, fragments from the pathogen and from the plant cuticle can be released and potentially detected more rapidly and/or be efficiently recognized by the plant as MAMPs and DAMPs, leading to the establishment of plant innate immunity. In several plant species, PRRs have been described as recognizing fungus-derived

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MAMPs, including CERK1, LYK4, LYM2, and CEBiP, which recognized chitin and EIX2 interacting with xylanase (reviewed in Zipfel, 2014). However, even if there is evidence that the plant can recognize cutin monomers as DAMPs and afterwards induce the defense responses against fungal pathogens (Schweizer *et al.*, 1994), to our knowledge, the molecular mechanism that recognizes cuticular components and triggers the defense response still has to been identified. In order to resolve this, we are currently screening for Arabidopsis mutants that no longer respond to cutin monomers (Aragón and Serrano, unpublished).

Future biotechnological perspectives

Since the cuticle is involved in the regulation of multiple biotic and abiotic interactions, its characterization and modification might lead to broad biotechnological applications. Here we describe some of them, including crop improvement, by modification of cuticle biochemistry, changes in the phyllosphere, and production of metabolites that potentially can regulate plant–microbe interactions.

In order to identify how the grape berry cuticle modifies agronomically related traits, 42 cultivars were physiologically and biochemically characterized. From this analysis, several plants were identified as showing increased resistance to *B. cinerea*, and this phenotype was directly related to the berry surface biochemistry, in particular to the content of cutin and wax (Gabler *et al.*, 2003). Interestingly, using a sensor-based method, measuring the electrical impedance of the grape berry cuticles and its epicuticular waxes, it was possible to confirm the correlation with resistance against *B. cinerea*, and to have a robust platform to identify varieties resistant to this pathogen (Herzog *et al.*, 2015). Additionally, using tomato fruit as a model, researchers have found a link between cuticle function and different phenotypes, including water loss/uptake, protection against UV radiation, mechanical support, self-cleaning surfaces, and resistance to pathogens (Martin and Rose, 2014). All this information, generated by both traditional and new tools, based on agronomically related traits, can now be used by plant breeders to select new varieties using marker-assisted selection (Yadav *et al.*, 2017).

The phyllosphere is formed by different kinds of microbes, including prokaryotes and eukaryotes such as bacteria and fungi, oomycetes and other protists. These plant-associated microorganisms have been described as extending beneficial traits to their host, including resistance to biotic and to abiotic stresses, uptake of nutrients and water, and triggering hormone-induced plant growth (Partida-Martínez and Heil, 2011; Bulgarelli *et al.*, 2013). Recently, Vogel *et al.* (2016) identified the protective ability of epiphytic non-pathogenic bacteria such as *Sphingomonas melonis* Fr1 (S.Fr1) and *Methylobacterium extorquens* PA1 against *Pseudomonas syringae* DC3000 (Fig. 1B) and found that this protection is related to the induction of plant defense response genes (Vogel *et al.*, 2016). Indeed, Innerebner *et al.* (2011) showed that members of the genus *Sphingomonas* also confer protection against the pathogen *P. syringae* DC3000, suggesting that substrate competition plays a role in plant protection by these bacteria, although an antibiosis mechanism was not excluded (Fig. 1B). Also as a side effect, the environment can be recovered by the degradation, detoxification and sequestration of pollutants mediated by these plant-resident microorganisms (Weyens *et al.*, 2015). Additionally, it has been described that changes in cuticle composition have a direct effect on the plant phyllosphere (Bodenhausen *et al.*, 2014). For instance, analysing the phyllosphere of cuticular mutants *bdg* and *lacs2* determined that microbes on the plant surface are important factors for plant resistance against the fungal pathogen *B. cinerea* (Ritpitakphong *et al.*, 2016). Based on this information, future plant breeding programs should be supported by close analysis of host–phyllosphere interactions, in particular the changes induced by cuticular composition (Kroll *et al.*, 2017).

Finally, Arabidopsis mutants (Table 1) with a modified cuticle have been shown to release diffusates that inhibit the development of *B. cinerea* on susceptible plants (Bessire *et al.*, 2007; Chassot *et al.*, 2007; Benikhlef *et al.*, 2013), though the biochemical nature of these diffusates is unknown. In order to identify the commonly presented metabolites in the diffusates that might lead to the resistance against *B. cinerea*, a metabolomic analysis was performed and 174 compounds were identified. Identification and characterization of these diffusates can lead to biotechnological applications against this important pathogen resident on the cuticle. However, whether these chemicals cause the inhibition of fungal growth is under investigation (L'Haridon and Serrano, unpublished data). To our knowledge, there is no evidence that other plants with a modified cuticle can release similar compounds and extend similar protection to *B. cinerea* or other microorganism and this deserves further investigation.

Conclusion

Summarizing, here we have described the important role of cutin monomers and waxes as signaling molecules during initial bacteria–plant and fungi–plant interactions. A few studies have shown that cuticular components are essential and important drivers of the bacterial community structure and size. On the other hand, cuticular components are also important for both pathogens and plants, to initiate the pre-invasion and infection process and to activate the innate immune response, respectively. Nevertheless, we are far from fully understanding these interactions. Future research will be needed to identify exactly which cuticular components are acting as signal molecules, how they are recognized, and how they are able to modify the development of the plant and the microorganisms. The answers to these questions will give us an insight into their use in plant breeding programs and other biotechnological applications.

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CAPÍTULO III.
Artículo publicado.

En este capítulo se presenta el artículo publicado con los resultados más importantes, producto de este trabajo doctoral, dentro de la edición especial: **Plant cuticle: from biosynthesis to ecological functions**, en la revista *Frontiers in plant science.* En este mismo capítulo se hace un resumen de los resultados publicados.

Arabidopsis thaliana cuticle composition contributes to differential defense response to *Botrytis cinerea*.

Aragón, W., Formey, D., Aviles-Baltazar, N. Y., Torres, M., and Serrano, M. (2021). *Frontiers in plant science*, 12, 738949**.**

III.1 Resumen de los resultados principales publicados en el artículo.

En este capítulo se muestran los principales resultados que respondieron a las preguntas de investigación de este trabajo doctoral y que fueron publicados. Se investigó el papel de los componentes de la cutícula vegetal como, cutina y ceras para desencadenar mecanismos asociados a la inmunidad innata ante la presencia del hongo fitopatógeno *B. cinerea*. Como modelo de estudio se caracterizaron a las mutantes de Arabidopsis afectadas solo en el contenido de cutina, como *bdg* y *lacs2-3;* afectadas solo en el contenido de ceras, como *cer1-4* y *cer3-6;* o afectada en el contenido de ambos componentes, como *eca2,* durante la interacción con *B. cinerea*.

Se demostró que solo las mutantes afectadas en el contenido de cutina, mostraron resistencia hacia el fitopatógeno (Aragón et al., 2021; Figura 1a-c); sin embargo, todas las mutantes presentaron una cutícula más permeable hacia el agua y a solutos; al igual que niveles altos de ROS (mayor intensidad en la fluorescencia y tinción), comparadas con sus respectivas plantas tipo silvestre (Aragón et al., 2021; Figuras 2 y 3). Interesantemente, aunque se observó mayor permeabilidad de la cutícula y acumulación de ROS en las mutantes afectadas en el contenido de ceras, el fenotipo de susceptibilidad observado (Aragón et al., 2021; Figuras 1-3) sugiere que estos cambios, no tienen una correlación directa con la resistencia que presentaron las mutantes afectadas en el contenido de cutina hacia *B. cinerea*, como se había propuesto en otros estudios.

Por lo anterior, para indagar en las bases moleculares que podrían contribuir a la respuesta de resistencia o susceptibilidad observada hacia *B. cinerea*, entre las mutantes afectadas en los componentes cuticulares, se realizó un análisis transcriptómico (RNA-seq) antes y después de la interacción con el fitopatógeno. De manera contrastante, durante la interacción, las mutantes afectadas en el contenido de ceras, comparten la inducción de genes asociados a estímulos abióticos (Aragón et al., 2021; Figura 6). Por su parte, a nivel individual, en las mutantes afectadas en el contenido de cutina, se identificó la inducción de genes relacionados con el estrés biótico, principalmente con las vías de síntesis de fitohormonas y con mecanismos de defensa canónicos (Aragón et

al., 2021; Figura 6). Sin embargo, entre los genes compartidos e inducidos en las mutantes afectadas en el contenido de cutina, resultó interesante que entre ellos no se identificaron genes involucrados en mecanismos de defensa canónicos contra *B. cinerea*; por el contrario, se identificaron genes involucrados en el remodelamiento de la pared celular como, *PME17* (AT2G45220) y *PME41* (AT4G02330), que codifican para pectin-metil-esterasas; así como genes que codifican para una catalasa *CAT3* y una peroxidasa *PRX71* (Aragón et al., 2021; Figura 8); los cuales han sido recientemente identificados como parte de las respuestas de defensa ante *B. cinerea* en interacción con plantas tipo silvestre de Arabidopsis. Asimismo, se identificó la inducción de genes que codifican para factores de transcripción, como *RAP2.6*/*ERF108* y *RAP2.6L*/*ERF113*; el gen *STP13* que codifica para una proteína transportadora de azúcares; y genes que codifican para receptores de reconocimiento de patrones (PRR) como, *RLK7*, *RLK5* y *RLP30* (Aragón et al., 2021; Figura 8). Estos genes resultaron interesantes, dado que no habían sido reportados previamente en mutantes afectadas en la composición de la cutícula, dentro del set de genes de defensa contra *B. cinerea.* Sugiriendo entonces, que la resistencia observada en las mutantes afectadas en el contenido de cutina, puede ser consecuencia de la activación de mecanismos de defensa distintos a los canónicos, además de la producción de ROS y de presentar una cutícula permeable.

En general, a pesar de los datos previos de algunas mutantes afectadas en la síntesis y regulación de las ceras cuticulares, en interacción con otros hongos patógenos, el vínculo entre la composición de las ceras y la resistencia o susceptibilidad hacia *B. cinerea* no se había descrito, así como tampoco los mecanismos moleculares involucrados. Por ello, en esta parte del trabajo doctoral, la información obtenida tanto para las mutantes afectadas en el contenido de ceras, como para las afectadas en el contenido de cutina, resulta valiosa y se puede utilizar como punto de partida para entender las bases moleculares involucradas en los primeros mecanismos de defensa relacionados con los componentes cuticulares contra este importante fitopatógeno agronómico.

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Arabidopsis thaliana Cuticle Composition Contributes to Differential Defense Response to *Botrytis cinerea*

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The chemical composition of a plant cuticle can change in response to various abiotic or biotic stresses and plays essential functions in disease resistance responses. *Arabidopsis thaliana* mutants altered in cutin content are resistant to *Botrytis cinerea*, presumably because of increased cuticular water and solute permeability, allowing for faster induction of defense responses. Within this context, our knowledge of wax mutants is limited against this pathogen. We tested the contribution of cuticular components to immunity to *B. cinerea* using mutants altered in either cutin or wax alone, or in both cutin and wax contents. We found that even all the tested mutants showed increased permeability and reactive oxygen species (ROS) accumulation in comparison with wild-type plants and that only cutin mutants showed resistance. To elucidate the early molecular mechanisms underlying cuticle-related immunity, we performed a transcriptomic analysis. A set of upregulated genes involved in cell wall integrity and accumulation of ROS were shared by the cutin mutants *bdg*, *lacs2-3*, and *eca2*, but not by the wax mutants *cer1-4* and *cer3-6*. Interestingly, these genes have recently been shown to be required in *B. cinerea* resistance. In contrast, we found the induction of genes involved in abiotic stress shared by the two wax mutants. Our study reveals new insight that the faster recognition of a pathogen by changes in cuticular permeability is not enough to induce resistance to *B. cinerea,* as has previously been hypothesized. In addition, our data suggest that mutants with resistant phenotype can activate other defense pathways, different from those canonical immune ones.

Keywords: cuticle, cuticular mutants, *B. cinerea*, permeability, ROS, cell wall

INTRODUCTION

A cuticle is a hydrophobic structure that covers the surface of the epidermal cells of the aerial parts of plants, such as leaves, stems, flowers, seeds, and fruits; and represents one of the evolutionary adaptations that has allowed plants to counteract the adverse effects produced by biotic and abiotic factors (Jeffree, 2006; Jetter et al., 2006; Riederer, 2006; Nawrath et al., 2013). The structure and chemical composition of a cuticle vary widely among different plant species, and even between

organs and stages of its development (Jeffree, 2006; Nawrath et al., 2013; Ingram and Nawrath, 2017). Despite this variability, all cuticles are mainly made up of two types of lipid compounds: cutin and waxes. Cutin is a polymer layer formed by a network of esterified ω-hydroxylated fatty acids *via* intermolecular ester bonds, leading to a three-dimensional structure that is produced and secreted by epidermal cells. Waxes comprise a mixture of very long-chain fatty acids (VLCFAs, 24–36 carbon atoms) and their derivatives, including alkanes, alcohols, and aldehydes, together with secondary metabolites, such as flavonoids and triterpenoids (Li-Beisson et al., 2013; Nawrath et al., 2013; Fernández et al., 2016).

Genetic approaches that use mutagenized populations of *Arabidopsis thaliana* (Bernard and Joubès, 2013; Yeats and Rose, 2013; Borisjuk et al., 2014; Domínguez et al., 2015; Fich et al., 2016), tomato, and maize (Isaacson et al., 2009; Javelle et al., 2010; Girard et al., 2012) have allowed for the identification of many key enzymes involved in cuticle biosynthesis and deposition. Some of these mutants, such as the cutin mutants *bodyguard* (*bdg*), *lacs2*, *lacerata* (*lcr*/*cyp86a8*), *cyp86a2*/*att1*, *abcg32*/*pec1*, and *myb96* (Wellesen et al., 2001; Schnurr et al., 2004; Xiao et al., 2004; Kurdyukov et al., 2006; Seo and Park, 2010; Benikhlef et al., 2013; Fabre et al., 2016; Zhao et al., 2019), and the wax mutants *fiddlehead* (*fdh*/*kcs10*), *cer1*, *cer3*/*wax2*, and *dewax* (Yephremov et al., 1999; Chen et al., 2003; Kurata et al., 2003; Rowland et al., 2007; Voisin et al., 2009; Sakuradani et al., 2013; Go et al., 2014; Liu et al., 2020) show a strong reduction in cutin and wax contents. Despite the loss of its cuticular structure, which might be thought to be detrimental to the plant, these mutants can accumulate significantly either more cutin monomers or more wax components when the other is reduced relative to wild type as a compensatory mechanism to maintain the integrity of cuticle (Voisin et al., 2009; Nawrath et al., 2013; Serrano et al., 2014).

To date, studies have identified the role of cuticular components during interaction with pathogens, showing that a cuticle is a physical and chemical barrier and that its components may act as signaling and defense molecules for both fungi and plants (Serrano et al., 2014; Aragón et al., 2017; Ziv et al., 2018). For instance, cutin monomers induce the germination of *Magnaporthe grisea* during the infection process of rice (*Oryza sativa*) (Gilbert et al., 1996), appressorium formation of the powdery mildew *Erysiphe graminis*in barley (*Hordeum vulgare)* (Francis et al., 1996), and induction of a protein kinase-mediated pathway required for the pathogenic development of *Colletotrichum trifolii* (Dickman et al., 2003). Besides the cutin monomers, specific wax components, such as very-long-chain (VLC) aldehydes, induce the pre-penetration process of *Blumeria graminis* both *in vitro* (Hansjakob et al., 2010) and *in planta* (Hansjakob et al., 2011). Additionally, other wax components, such as VLC primary alcohols of avocado (*Persea americana*), induce germination and appressorium formation of *Colletotrichum gloeosporioides* (Podila et al., 1993). In contrast, plants might perceive cutin monomers released by the action of fungal cutinase as elicitors. This hypothesis was evaluated when rice and barley plants were treated with synthetic cutin monomers (C18 fatty acids) and showed resistance to *E. graminis* and *M. grisea,* respectively (Schweizer et al., 1996). In the same way, cucumber seedlings respond to hydrolyzates of cutin by producing H_2O_2, which has been associated with early defense responses against pathogens (Fauth et al., 1998).

Cuticular mutants and transgenic lines have contributed to the advancement of our knowledge of how defects in cuticle structure might lead to immunity of plants upon the attack by pathogenic fungi. Fungal cutinase-expressing (CUTE) plants and *A. thaliana* cutin mutants with an altered ultrastructure and increased permeability of the cuticle, such as *lacs2* (deficient in the long-chain acyl-CoA synthetase 2 enzyme that catalyzes the synthesis of intermediates in the cutin pathway and in wax biosynthesis), *bdg* (mutated in BODYGUARD, an extracellular α/β hydrolase suggested to be involved in cutin polyester assembly), and *lcr* (mutated in *CYP86A8*, which is involved in the biosynthesis of cutin pathway), displayed increased resistance to the necrotrophic fungus *Botrytis cinerea* (Sieber et al., 2000; Bessire et al., 2007; Chassot et al., 2007; Tang et al., 2007; Voisin et al., 2009). Likewise, we have recently described that a mutant with a strong reduction in both cutin and wax contents, *eca2* (expression constitutiva del gen*ATL2)*, is resistant to *B. cinerea* and to the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv tomato strain DC3000 (*Pst* DC3000), but susceptible to *Phytophthora brassicae* compared with WT plants (Blanc et al., 2018). In contrast, the mutants *lacs2,acp4,* and *myb96* with altered cutin content exhibited enhanced susceptibility against *Pst* DC3000 (Tang et al., 2007; Xia et al., 2009; Seo and Park, 2010). Besides these cutin mutants, only a few *A. thaliana* mutants with defects in wax biosynthesis or regulation have been screened for their responses to different pathogens. For instance, *cer1-1* mutants affected in *CER1* (wax biosynthetic gene fora VLC-aldehyde decarbonylase) have a significantly reduced wax load, showed susceptibility to the necrotrophic fungus *Sclerotinia sclerotiorum* (Aarts et al., 1995; Bourdenx et al., 2011), and enhanced resistance to the biotrophic fungus *Golovinomyces orontii*, whereas, in *cer3-6* and *cer3-8* mutants affected in *CER3/WAX2/YRE* (a wax biosynthetic gene for VLC-acyl-CoA reductase), the growth and reproduction of these fungi were slightly inhibited (Rowland et al., 2007; Inada and Savory, 2011). An evaluation of *in planta* bacterial growth in *cer1-1* confirmed susceptibility to bacterium *Pst* DC3000 (Xia et al., 2009; Bourdenx et al., 2011), as well as in the mutant *cer3-6* (Lee et al., 2016). The *dewax* mutant (knockout in *DEWAX* that codified to the transcription factor DEWAX, which represses cuticular wax biosynthesis) has been reported to be more susceptible to *B. cinerea* (Go et al., 2014; Ju et al., 2017). In order to explain the resistance against *B. cinerea* observed on mutants with altered cutin composition, several reports have characterized physiological changes and the induction of defense responses. These reports include analysis on cuticular water and solute permeability (hereafter referred to as cuticular permeability), the production of reactive oxygen species (ROS), expression of genes implicated in plant defense signaling pathways (Bessire et al., 2007; Chassot et al., 2007; Voisin et al., 2009; L'Haridon et al., 2011; Blanc et al., 2018), and analysis of the abscisic acid (ABA) signaling pathway (L'Haridon et al., 2011; Cui et al., 2016). Based on these reports, a model to explain the cuticle-derived resistance to *B. cinerea* was proposed. In the

cutin mutants, changes in cuticular structure and permeability allow pathogen- and/or damage-associated molecular patterns (PAMPs or DAMPs), released from both the pathogen and the plant cuticle or cell wall, respectively, to be more rapidly recognized by plant pattern recognition receptors, triggering immune responses (Serrano et al., 2014). Nevertheless, we are far away from fully understanding the early mechanisms and role(s) that cuticular components might play during the plant-fungal pathogen interaction, especially against *B. cinerea*, which leads to resistance.

In this report, we characterized mutants altered in either cutin (*bdg*, *lacs2-3*) or wax (*cer1-4* and *cer3-6*) alone, or altered in both cutin and wax (*eca2*) contents during the interaction with *B. cinerea*. We determined that while all the mutants have an increased permeability, only the cutin mutants were resistant to this pathogen. Additionally, in order to identify the molecular elements that lead to this resistance or susceptibility, we performed a genome-wide transcriptional characterization before and after the challenge with the fungus. This analysis allowed us to identify a set of genes, expressed only in mutants altered in cutin content, that have recently been described as part of resistance mechanisms against *B. cinerea* (Lionetti et al., 2017; Bacete et al., 2018; Del Corpo et al., 2020). Our study allows us to understand how modification in cuticular components activates defense responses against this agronomical important phytopathogen.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana plants were grown in a greenhouse at 22 to 23°C and 60% humidity under a long day photo period (16-h light) for 4 weeks. The following plants were used: C24 ecotype as wild-type (WT) for the *eca2* mutant altered in both cutin and wax components (Salinas-Mondragón et al., 1999; Serrano and Guzmán, 2004; Blanc et al., 2018) and Columbia-0 (Col-0) as WT for mutants altered in cutin content: *bdg* (Kurdyukov et al., 2006; Voisin et al., 2009) and *lacs2-3* (CS65776 (obtained from the Arabidopsis Biological Resource Center, ABRC) (Bessire et al., 2007). The mutants altered in wax content were *cer1-4* (SALK_008544C) (Bourdenx et al., 2011) and *cer3-6* (*yre-1*) (Rowland et al., 2007), and were kindly provided by Professor Ljerka Kunst, Department of Botany, University of British Columbia, Vancouver, BC, Canada. All the selected mutants have been genetically (confirmed homozygous lines) and chemically characterized in detail (**Supplementary Table 1**).

Pathogen Infection Assays

Botrytis cinerea strain B05.10 was cultured on potato dextrose agar (PDA, 39 g L^{-1}) plates. Spores were harvested in distilled water and filtered to remove hyphae. For inoculations, spore concentration was adjusted to 5×10^4 spores ml^{-1} in 1/4 - strength potato dextrose broth (PDB, 6 g L^{-1}; Sigma-Aldrich, United States). For the analysis of lesion development, six fully expanded leaves per 4-week-old soil-grown plant were inoculated with a single drop of 6 μl of a spore suspension over each leaf, and at least 30 lesions were evaluated in each experiment. The inoculated plants were covered with plastic lids to maintain moisture level and transferred to a growth chamber at 22°C and a 24-h dark cycle. After 72 hpi, symptoms were evaluated. The level of resistance (disease incidence) was expressed by the percentage of plants showing disease symptoms extending beyond the inoculation site in each mutant. The developed lesions were quantified using the Image J analysis software (Fiji Is Just Image J[1]) (Schindelin et al., 2012). The experiments were repeated with at least three individual biological replicates, each with 10 technical replicates.

Cuticular Permeability Assays

The toluidine blue staining performed was from a previously described method (Tanaka et al., 2004; Bessire et al., 2007). Rosette leaves of 4-week-old plants were detached and immersed for 2 h in 0.025% TB (Sigma-Aldrich, United States) solution in 1/4 PDB (Sigma-Aldrich, United States) and were rinsed with tap water. Photos were used to measure the stained area (mm^2) using imageJ see text footnote 1. For staining with calcofluor white (Sigma-Aldrich, United States), the leaves were bleached in absolute ethanol overnight, incubated in 0.2 M sodium phosphate buffer (pH 9) for 1 h, and for 5 min in 0.5% calcofluor white in 0.2 M sodium phosphate buffer (pH 9). Then, the leaves were rinsed in sodium phosphate buffer to remove excess calcofluor solution, and photographed under UV light (L'Haridon et al., 2011). Chlorophyll leakage from the rosette leaves was determined by a previously described protocol (Schnurr et al., 2004). For chlorophyll measurement, the fresh weight of detached leaves of mutants and wild-type plants was measured, and they were immersed in 80% ethanol. After 1 h, 1-ml aliquots were removed, and absorbance was measured at 664 and 647 nm. The micromolar concentration of total chlorophyll per gram of fresh weight of leaves was calculated using the equation: total micromoles chlorophyll = 7.93 (A_{664}) + 19.53 (A_{647}) (Lolle et al., 1997; Voisin et al., 2009). The experiments were repeated with at least three biological replicates, each with six technical replicates.

Detection of Reactive Oxygen Species

3, 3′-Diaminobenzidine and NBT staining were performed to determine the presence of hydrogen peroxide (H_2O_2) and superoxide (O_2^-), respectively. The presence of H_2O_2 was visualized with 3, 3′-diaminobenzidine (Sigma-Aldrich, United States) (Thordal-Christensen et al., 1997; L'Haridon et al., 2011). Detached leaves were immersed in 1 mg ml^{-1} DAB-HCl, pH 3.8, by gentle vacuum infiltration. For superoxide (O_2^-) staining, detached leaves were immersed for 30 min in 0.1% nitroblue tetrazolium (NBT) chloride (Sigma-Aldrich, United States) in 50 mM potassium phosphate buffer pH 7.5 (L'Haridon et al., 2011; Lehmann et al., 2015). Following incubation, the DAB and NBT staining solutions were removed and replaced with a bleaching solution (ethanol: acetic acid: = 3:1). H_2O_2 was visualized as a reddish-brown stain formed by the reaction of DAB with endogenous H_2O_2. The O_2^- content was detected as a dark blue stain of a formazan compound formed as a result of

[1] https://imagej.net/Fiji

NBT reacting with endogenous O^{2-}. The reactive oxygen species (ROS) production in detached rosette leaves unchallenged and challenged with the pathogen was detected using 5-(6) carboxy-2′, 7′-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich, United States). The leaves were immersed in 60 μM of DCF-DA in a standard medium (1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM 2-morpholinoethanesulfonic acid adjusted to pH 6.1 with NaOH) (L'Haridon et al., 2011; Benikhlef et al., 2013). The leaves were then observed using a Carl Zeiss Axioplan 2 epifluorescence microscope with a GFP filter set (excitation 480/40 nm, emission 527/30 nm). Microscope images were saved as TIFF files, and the accumulation of fluorescence was quantified as pixels using imageJ see text footnote 1.

RNA Extraction, RNA Sequencing, and Analysis

For RNA-seq, rosette leaves of the Col-0, C24, *eca2*, *bdg*, *lacs2-3*, *cer1-4,* and *cer3-6* plants were inoculated by spraying the entire leaves with spore suspension (5×10^4 spores ml^{-1}) of *B. cinerea.* At least eight whole rosettes of each plant were collected at 6 hpi and as well under non-infected conditions. Total RNA for RNA-seq was isolated from two different biological replicates for each mutant and WT using a Spectrum™ Total RNA kit (Sigma-Aldrich, United States). Total RNA concentration and purity were measured using NanoDrop™ 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, United States). Library construction and sequencing were performed by Beijing Genomics Institute (BGI) Americas[2] using DNBSeq™ technology. The sequencing was performed using paired end generating 100-bp size reading. The sequences are publicly available in the following link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA761130?reviewer=jgq29l4gjk5tenf7e4q3755opm.

Approximately 20 million reads per sample were aligned to the *A. thaliana* transcriptome ([3]TAIR version 10) using Bowtie2 (v2.3.5) (Langmead and Salzberg, 2012). The bioinformatics data processing summary for each mutant is shown in **Supplementary Table 2**. We calculated gene expression levels using the RNA-seq by expectation maximization (RSEM) method (v1.3.3) (Li and Dewey, 2011). Differentially expressed genes (DEGs) were identified using the software DESeq2 in the *Integrated Differential Expression Analysis MultiEXperiment* (IDEAMEX) (Jiménez-Jacinto et al., 2019), with a FoldChange $\geq$ 2, and adjusted *p*-value $\leq$ 0.05. Additionally, the DEGs were functionally annotated with Gene Ontology (GO) terms extracted with PANTHER (v16.0) (GO term enrichment analysis) and Database for Annotation, Visualization, and Integrated Discovery (DAVID) (v6.8), by using Fisher's exact test and correction with an FDR. Plots were created with the ggplot2 library using RStudio (v1.4.1106). To further identify DEGs common among the cutin and wax mutants, we drew Venn diagrams using the VennDiagram package in R (v4.0.3), and the webtool Venn Diagram[4]. Figures showing heatmaps were generated using the webtool Heatmapper[5] (Babicki et al., 2016).

[2]https://www.bgi.com/us

[3]https://www.arabidopsis.org/

[4]http://bioinformatics.psb.ugent.be/webtools/Venn/

[5]http://www.heatmapper.ca/expression/

Quantitative RT-PCR Analysis

Total RNA was isolated from the frozen rosette leaves of WT and the mutants infected and non-infected with *B. cinerea* (6 hpi) collected directly into liquid nitrogen and stored at −80°C. Wet weight of 100 mg was used for RNA isolation from a pool of leaves from six plants of each genotype using TRI Reagent® (Sigma-Aldrich, United States), following the instructions of the manufacturer. Sample quality was assessed by using denaturing gel electrophoresis and measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, United States). A 1-μg sample of total RNA was treated with DNase I, RNAse-free (Thermo Fisher Scientific, Inc., Waltham, MA, United States), and then used as the template for cDNA synthesis with a RevertAid H Minus RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, United States). Quantitative RT-PCR (RT-qPCR) reactions contained cDNA (diluted 1/40) in Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific, Inc., Waltham, MA, United States) and 0.5 μM of specific primers. Primers for the RT-qPCR gene expression analysis have been previously described: *AtPME17* (Del Corpo et al., 2020), *AtPME41* (Choi B. et al., 2017), *RAP2.6/ERF108* (Imran et al., 2018), and *CAT3* (Zou et al., 2015). All the reactions were performed in 96-well plates using the 7300 Real-Time PCR System and 7300 System Software (Applied Biosystems, Foster City, CA, United States). PCR conditions were 95°C initial denaturations for 15 min, 40 cycles of 15 s/95°C, 30 s/60°C, and 30 s/72°C, after each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 60 to 95°C. The relative gene expression level for each sample was calculated using the comparative Ct method (Schmittgen and Livak, 2008) and normalized with the geometrical mean of two housekeeping genes, *CF150* (AT1G72150) and *ACT2* (AT3G18780) (Serrano and Guzmán, 2004; Czechowski et al., 2005). One-way ANOVA followed by Tukey comparisons was performed to evaluate the significance of the differential gene expression using the mean values from three biological replicates for each sample.

Statistical Analysis

One-way analysis of variance, followed by Tukey's (honestly significant difference (HSD) comparisons, was performed to determine statistical significance. GraphPad Prism8 v 8.0.1 (GraphPad Software, San Diego CA, United States) was used. Data represent the mean ± SE. Differences at $p < 0.001$ were considered significant.

RESULTS

Mutants With Alteration in Cuticular Wax or Cutin Composition Confer Differential Resistance to *Botrytis cinerea*

In recent years, a number of *Arabidopsis* mutants with defects in different steps of cutin biosynthesis, transgenic plants expressing a fungal cutinase and/or direct application of cutinase on wild type rosette leaves, have shown resistance to *B. cinerea*. These

results has been interpreted as evidence of the participation of the cutin monomers in the resistance against this pathogen (Sieber et al., 2000; Chassot et al., 2007; Voisin et al., 2009). Despite the previous data from several mutants involved in the synthesis and regulation of cuticular waxes, in interaction with pathogens (Bourdenx et al., 2011; Lee et al., 2016; Ju et al., 2017), the link between wax composition/structure and the resistance or susceptibility of mutants against *B. cinerea* have not been described in detail. In addition, molecular defense mechanisms underlying the phenotypes of both the wax and cutin mutant lines mainly affected in cuticular biosynthesis are still unknown. To determine if changes in wax composition lead to resistance to this phytopathogen, we confronted the *eca2* mutant (with modified cutin and wax components), cutin mutants *lacs2-3* and *bdg,* and two mutants with reduced wax content, *cer1-4* and *cer3-6,* with *B. cinerea* and compared them with their corresponding wild-type plants. After inoculation (3 dpi), we observed that only *eca2*, *bdg,* and *lacs2-3* showed resistance to this pathogen (**Figure 1A**). These cutin-altered mutants exhibited less than 20 and 35% of disease incidence, respectively, while their corresponding WT plants showed an incidence of 100% (**Figure 1B**). However, in the *cer1-4* and *cer3-6* mutants affected only in wax composition, susceptibility similar to that of Col-0 was observed (**Figures 1A,B**). Additionally, the lesion average area was significantly smaller in the cutin mutants *eca2,bdg,* and *lacs2-3* compared with the wax mutants *cer1-4* and *cer3-6* (**Figure 1C**). One interesting observation is that under our experimental conditions, the majority of leaves from *eca2* remained free of disease symptoms 7 dpi compared with their corresponding WT plants (C24), whereas only some of the leaves from *bdg* and *lacs2-3* remained free of disease symptoms at this time. In contrast, all the inoculated leaves of *cer1-4* and *cer3-6* already showed signs of fungal infection at 3 dpi, similar to the WT plants (data not shown). These results indicated that an altered wax composition does not correlate with resistance against the necrotrophic fungi *B. cinerea*, as previously observed in cutin mutants.

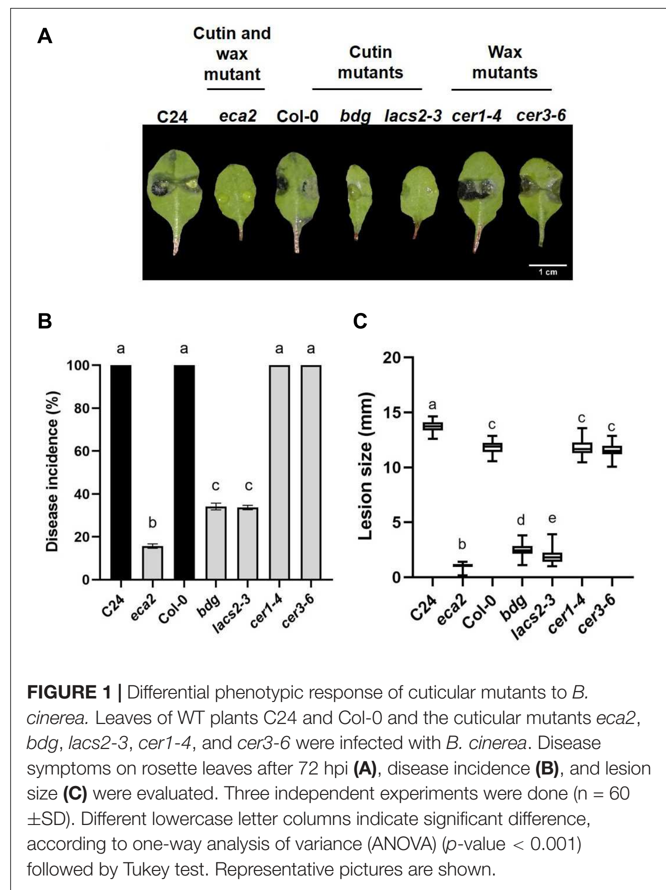

FIGURE 1 | Differential phenotypic response of cuticular mutants to *B. cinerea.* Leaves of WT plants C24 and Col-0 and the cuticular mutants *eca2*, *bdg*, *lacs2-3*, *cer1-4*, and *cer3-6* were infected with *B. cinerea*. Disease symptoms on rosette leaves after 72 hpi **(A)**, disease incidence **(B)**, and lesion size **(C)** were evaluated. Three independent experiments were done (n = 60 ±SD). Different lowercase letter columns indicate significant difference, according to one-way analysis of variance (ANOVA) (p-value < 0.001) followed by Tukey test. Representative pictures are shown.

Changes in Cutin or Wax Content Lead to Increased Leaf Permeability

Previous reports on the *bdg*, *lacs2-3,* and *eca2* mutants have shown that they have a permeable cuticle (Bessire et al., 2007; Chassot et al., 2007; Voisin et al., 2009; L'Haridon et al., 2011; Blanc et al., 2018). To determine if the wax mutants present similar permeability, we assessed it by toluidine blue (TB) and calcofluor staining and measuring the increased efflux of chlorophyll, as previously described (Tanaka et al., 2004; L'Haridon et al., 2011; Cui et al., 2016). The *eca2*, *bdg,* and *lacs2-3* leaves showed the characteristic dark blue and bright patterns correlated with the TB and calcofluor stain, respectively, while *cer1-4* and *cer3-6* had weaker calcofluor staining and smaller TB-stained area compared with the cutin mutants (**Figures 2A,B**). Nevertheless, the quantification of the TB-stained area shows that all the mutants (such as *cer1-4* and *cer3-6*) present statistically significant increased permeability compared with their corresponding WT plants (**Figure 2B**). Next, we analyzed cuticular permeability by chlorophyll leaching. When non-infected rosette leaves are immersed in 80% ethanol, mutants defective either in cutin and/or wax composition lose chlorophyll more rapidly than their corresponding WT plants (**Figure 2C**). We observed that the cutin mutants *lacs2-3* and *eca2* had similar chlorophyll-leaching rates of approximately 4-fold more than their corresponding WT plants. The *bdg*, *cer1-4* and *cer3-6* mutants show 3- and 2-fold greater chlorophyll leaching, respectively, compared with Col-0 (**Figure 2C**), thus corroborating the results of the toluidine blue and calcofluor tests. Taken together, these results indicate that modification of the content of cutin and/or wax leads to a more permeable cuticle.

Cuticle-Related Mutants Show Basal Reactive Oxygen Species Accumulation

Previous reports on *Arabidopsis* mutants with defects in cuticle structure associated with alterations in the composition of cutin monomers showed increased reactive oxygen species (ROS) levels, even when leaves were not challenged with a pathogen (Chassot et al., 2007; L'Haridon et al., 2011; Blanc et al., 2018). To test if the production of ROS, one of the most early and rapid defense reactions to pathogen attack, is present in mutants with alterations in the composition of cuticular wax, we evaluated the accumulation of ROS in uninfected leaves of *eca2, bdg*, *lacs2-3, cer1-4,* and *cer3-6* using three different dyes: 5-(and-6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCF-DA) that detects a broad range of oxidizing reagents (L'Haridon et al., 2011); 3, 3′-diaminobenzidine (DAB), which

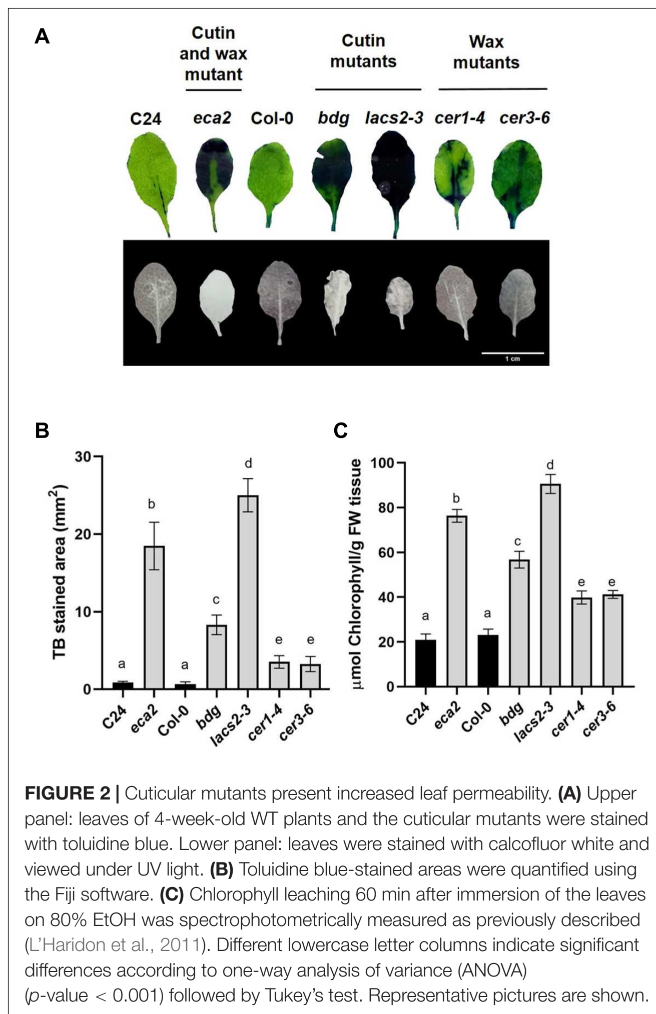

FIGURE 2 | Cuticular mutants present increased leaf permeability. **(A)** Upper panel: leaves of 4-week-old WT plants and the cuticular mutants were stained with toluidine blue. Lower panel: leaves were stained with calcofluor white and viewed under UV light. **(B)** Toluidine blue-stained areas were quantified using the Fiji software. **(C)** Chlorophyll leaching 60 min after immersion of the leaves on 80% EtOH was spectrophotometrically measured as previously described (L'Haridon et al., 2011). Different lowercase letter columns indicate significant differences according to one-way analysis of variance (ANOVA) (p-value < 0.001) followed by Tukey's test. Representative pictures are shown.

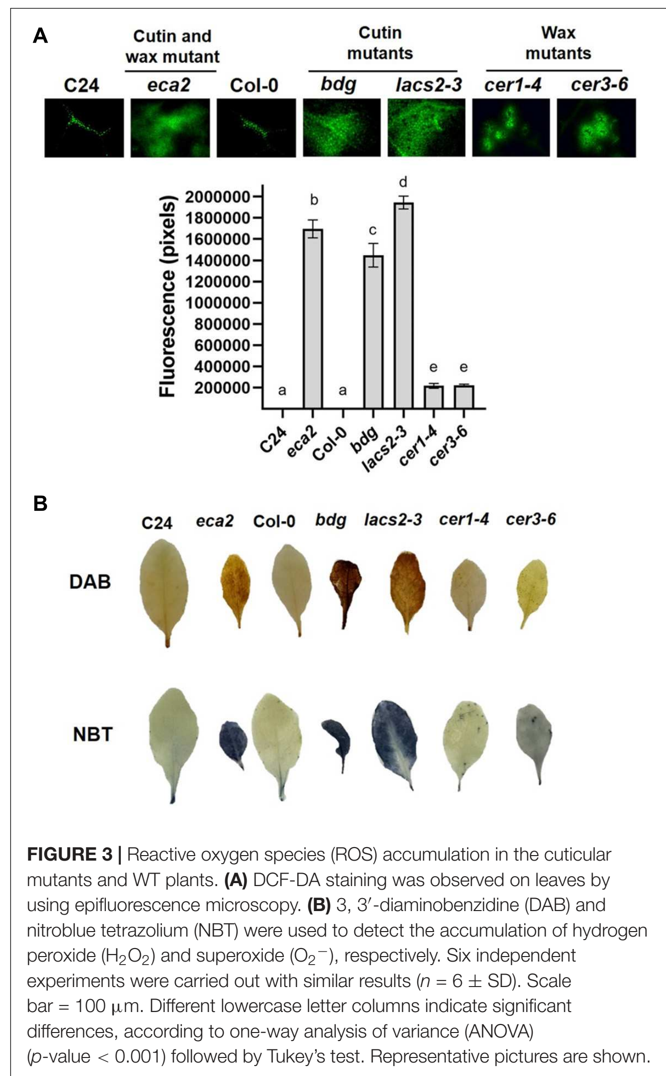

FIGURE 3 | Reactive oxygen species (ROS) accumulation in the cuticular mutants and WT plants. **(A)** DCF-DA staining was observed on leaves by using epifluorescence microscopy. **(B)** 3, 3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) were used to detect the accumulation of hydrogen peroxide (H_2O_2) and superoxide (O_2^-), respectively. Six independent experiments were carried out with similar results (n = 6 ± SD). Scale bar = 100 µm. Different lowercase letter columns indicate significant differences, according to one-way analysis of variance (ANOVA) (p-value < 0.001) followed by Tukey's test. Representative pictures are shown.

detects H_2O_2; and nitroblue tetrazolium (NBT), which detects O_2^-(Thordal-Christensen et al., 1997; Mengiste et al., 2003). Stronger DCF-DA fluorescence was observed in *eca2*, *bdg,* and *lacs2-3* compared with the mutants with altered wax content, *cer1-4* and *cer3-6* (**Figure 3A**). Nevertheless, ROS accumulation in the latter mutants was stronger than in Col-0 (**Figure 3A**). The DAB and NBT staining showed that coloration in the mutants with altered cutin (*eca2, bdg,* and *lacs2*) was much darker than in *cer1-4* and *cer3-6*, indicating higher ROS accumulation (**Figure 3B**). To further study this immune response, we analyzed ROS accumulation in leaves at 6 hpi with *B. cinerea*. We observed that all the mutants, such as *cer1-4* and *cer3-6,* showed stronger accumulation than their corresponding WT plants in all the staining methodologies (DCF-DA, DAB, and NBT) (**Supplementary Figure 1**). At this point, our data illustrate a possible correlation between cuticle permeability, ROS production, and *B. cinerea* resistance associated with alterations in the composition of cutin monomers, as previously described. However, although cuticle permeability and ROS accumulation are observed in the wax mutants, their susceptibility suggests that these changes might not only be associated with the resistance against *B. cinerea*, as we have previously hypothesized.

Reduction in Cutin and Wax Contents Induced Differential Transcriptional Changes

To investigate the molecular basis that might contribute to the differential response against *B. cinerea* among the cuticular mutants, RNA transcriptome sequencing (RNA-seq) and analysis were performed on non-infected plants. We identified that the number of differentially expressed genes (DEGs) was different in each mutant, compared with their corresponding WT plant, as follows: 2,386 in *eca2*, 3,506 in *bdg*, 1,688 in *lacs2-3*, 2,819 in *cer1-4,* and 611 in *cer3-6* (**Figure 4A** and **Supplementary Data 1**). Additionally, we studied if these DEGs were shared among the mutants (**Figure 4B**). Interestingly, a large proportion of the DEGs (up- and down-regulated genes) was unique in most of the mutants, except for *lacs2-3* and *cer3-6*. For instance, 68, 51, and 69% of the DEGs were only detected in *eca2*, *bdg,* and *cer1-4,* respectively, while 35 and 23% of the DEGs were only identified in *lacs2-3* and *cer3-6* (**Figure 4B**). We also looked at DEGs shared only

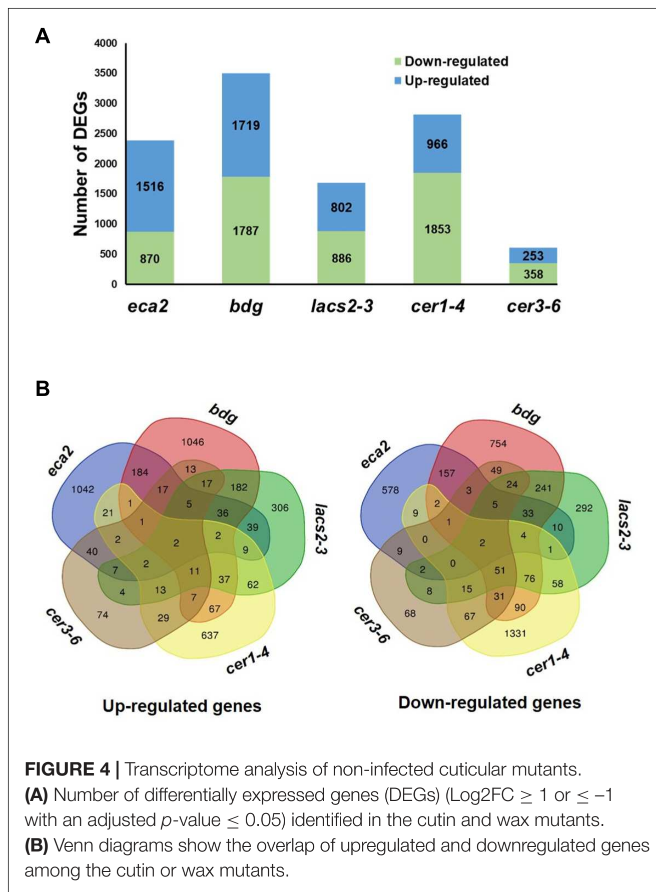

FIGURE 4 | Transcriptome analysis of non-infected cuticular mutants. **(A)** Number of differentially expressed genes (DEGs) (Log2FC ≥ 1 or ≤ −1 with an adjusted *p*-value ≤ 0.05) identified in the cutin and wax mutants. **(B)** Venn diagrams show the overlap of upregulated and downregulated genes among the cutin or wax mutants.

in *B. cinerea*-resistant or susceptible mutants (**Supplementary Data 2**). The Gene Ontology (GO) analysis of these DEGs from the cutin and wax mutants showed substantial differences. Among the 36 induced genes identified in the three cutin mutants, there is enrichment in the transmembrane receptor protein tyrosine kinase signaling pathway, wax biosynthetic process, and cuticle development. Among the 29 induced genes in the wax mutants, there is enrichment in the regulation of response to stress, regulation of response to external stimulus, and developmental process involved in reproduction (**Supplementary Data 3**). On the other hand, for 67 genes that are downregulated in the wax mutants, the DEG biological process categories were associated with photosynthesis and response to abiotic stress, while the 33 downregulated genes identified in the cutin mutants were classified as regulation of metabolic process (**Supplementary Data 3**). Finally, we identified that only two genes were upregulated in all five mutants. They were *GRP3* (AT2G05520), which encodes an Arabidopsis Glycine Rich Protein, and the AT3G14470, which encodes an NB-ARC domain-containing disease resistance protein. Similarly, only two genes were downregulated in all the five mutants: *CBF3* (AT4G25480), which encodes a member of the ERF/AP2 transcription factor family, and a gene that encodes a xyloglucan endotransglycosylase/hydrolase (AT3G44990) (**Figure 4B**). These results suggest a differential transcriptional regulation in all the cuticular mutants.

Under Non-challenged Conditions, Cutin Mutants Transcriptionally Induced Differential Defense Responses

A transcriptional modification of plant defense response genes in non-infected cutin mutants has been previously described (Voisin et al., 2009; Nawrath et al., 2013). However, to our knowledge, it has not been shown in wax mutants. The GO analysis on the identified DEGs for each mutant reveals that only *eca2*, *bdg,* and *lacs2-3* show the modification of expression of genes classified as part of the response to biotic stresses (**Figure 5** and **Supplementary Data 4**). These GO processes included: response to biotic stimulus and response to other organisms, involved in interspecies interaction between organisms and defense responses, while in the mutants *cer1-4* and *cer3-6*, statistically significant GO processes were classified into the response to abiotic stresses (**Figure 5** and **Supplementary Data 4**). In order to further characterize these results in cutin mutants, we analyzed the transcriptome profile of selected marker genes related to the jasmonic acid/ethylene- (JA/ET), salicylic acid (SA), and abscisic acid (ABA) pathways that have been described to be induced during the interaction with this necrotrophic pathogen (AbuQamar et al., 2006; Windram et al., 2012) (**Supplementary Figure 2**, **Supplementary Data 1**). Interestingly, under non-infected conditions, most of these genes were actually downregulated and only a few were induced. These results suggest that while the basal transcriptomic response to biotic stresses is activated in the cutin mutants, canonical defense responses against this pathogen are not induced before the interaction occurs.

Plant Defense Response Genes Are Induced Only in Cutin Mutants During the Interaction With the Pathogen

To further study the modification of the plant transcriptome in cutin and wax mutants, we performed RNA-seq analysis of *B. cinerea*-infected *Arabidopsis* leaves at 6 hpi. Interestingly, we observed a clear difference in the number of genes that are induced or repressed in the cutin mutants compared with the wax mutants. For instance, the number of DEGs in *eca2*, *bdg,* and *lacs2-3* was 2,595, 4,823, and 4,865; while in *cer1-4* and *cer3-6,* the number was only 241 and 411, respectively (**Figure 6A**, **Supplementary Data 5**). This represents approximately 10- to 8-fold more DEGs in the cutin mutants than in the mutants with reduced content of wax. To determine the processes that are transcriptionally induced, we performed a GO analysis of upregulated genes (**Figure 6C**). This analysis reveals a clear enrichment of GO terms related to response to a biotic stimulus only in the mutants with a reduced level of cutin. These terms include the biological process involved in interspecies interaction between organisms, defense response, response to biotic stimulus, response to fungus, and defense response to other organisms, among others (**Figure 6C**, **Supplementary Data 6**), while the GO analysis of upregulated genes in *cer1-4* and *cer3-6* reveals enrichment in response to abiotic stimuli, such as response to an organic substance, cytokinin-activated

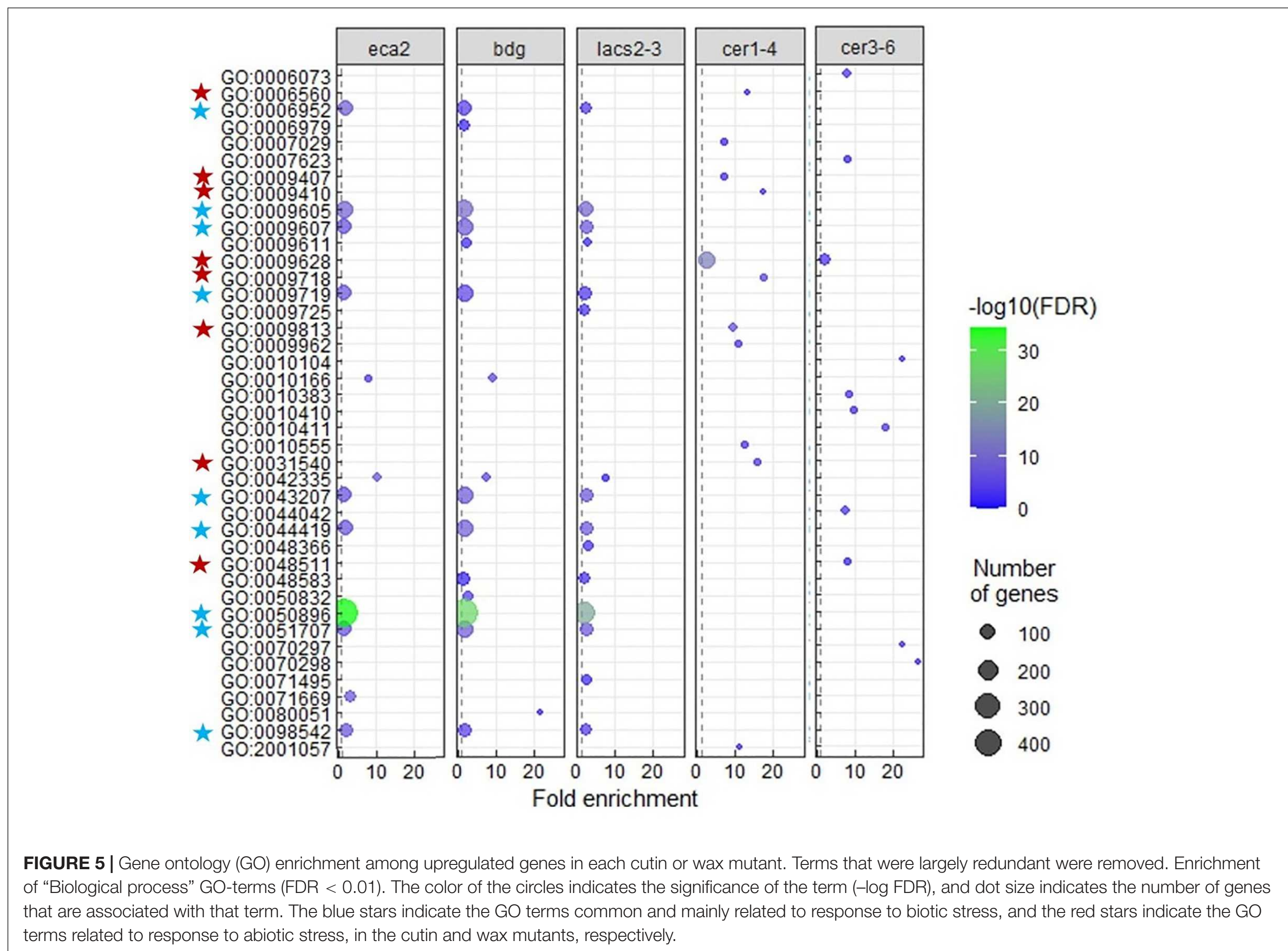

FIGURE 5 | Gene ontology (GO) enrichment among upregulated genes in each cutin or wax mutant. Terms that were largely redundant were removed. Enrichment of "Biological process" GO-terms (FDR < 0.01). The color of the circles indicates the significance of the term (–log FDR), and dot size indicates the number of genes that are associated with that term. The blue stars indicate the GO terms common and mainly related to response to biotic stress, and the red stars indicate the GO terms related to response to abiotic stress, in the cutin and wax mutants, respectively.

signaling pathway, response to chemical, and response to stimulus (**Figure 6C**, **Supplementary Data 7**). Additionally, we found that 214 and 11 DEGs were commonly induced in the cutin and wax mutants, respectively (**Figure 6B**, **Supplementary Data 7**). Additionally, 240 common downregulated genes were identified in *eca2*, *bdg,* and *lacs2-3* (**Figure 6B**, **Supplementary Data 7**), which were classified into the following GO terms: response to salt stress, response to oxidative stress, response to water deprivation, and response to cold. The seven repressed genes common between *cer3-6* and *cer1-4* belong to response to stimulus, single organism process, and response to external stimulus (**Supplementary Data 8**). Taken together, these data indicate that the enhanced resistance against *B. cinerea* observed in *eca2*, *bdg,* and *lac2-3* could be explained by the expression of defense-related genes, which are not induced in the wax mutants.

Canonical Defense Response Genes Are Differentially Induced in Cutin Mutants

We further characterized the defense mechanisms in the mutants with reduced levels of cutin that might participate in the resistance against *B. cinerea*. We were interested in identifying if JA/ ET-, SA-, ABA- and/or other defense-related genes were differentially expressed in the cutin and wax mutants (**Figure 7**). Remarkably, even though many of the hormone- and defense-related genes were induced in all the cutin mutants, we did not detect genes that were expressed simultaneously in all of them, except for the LRR receptor-like protein kinase (*BAK1*) and its interactor *Botrytis-induced kinase 1* (*BIK1*), which are involved in the early stages of recognition of pathogens (Veronese et al., 2005; Liu et al., 2017; van der Burgh et al., 2019). This result suggests that resistance against *B. cinerea* might not be exclusively mediated by these well-described genes.

A Set of Genes Related to Cell Wall Remodeling Is Induced in Mutants With Altered Cutin Monomer Content

Based on the expression profile of *BAK1* and *BIK1*, we identified all the genes that are commonly upregulated among the three cutin resistant mutants, *eca2*, *bdg,* and *lacs2-3,* but downregulated in *cer1-4* and *cer3-6* (**Figures 6B**, **8A**). We identified 214 genes that share this expression pattern (**Supplementary Data 7**). From this set of genes, we performed a GO analysis and the genes were classified into the response to biotic stimulus, response to fungus, innate immune response, and defense response to other

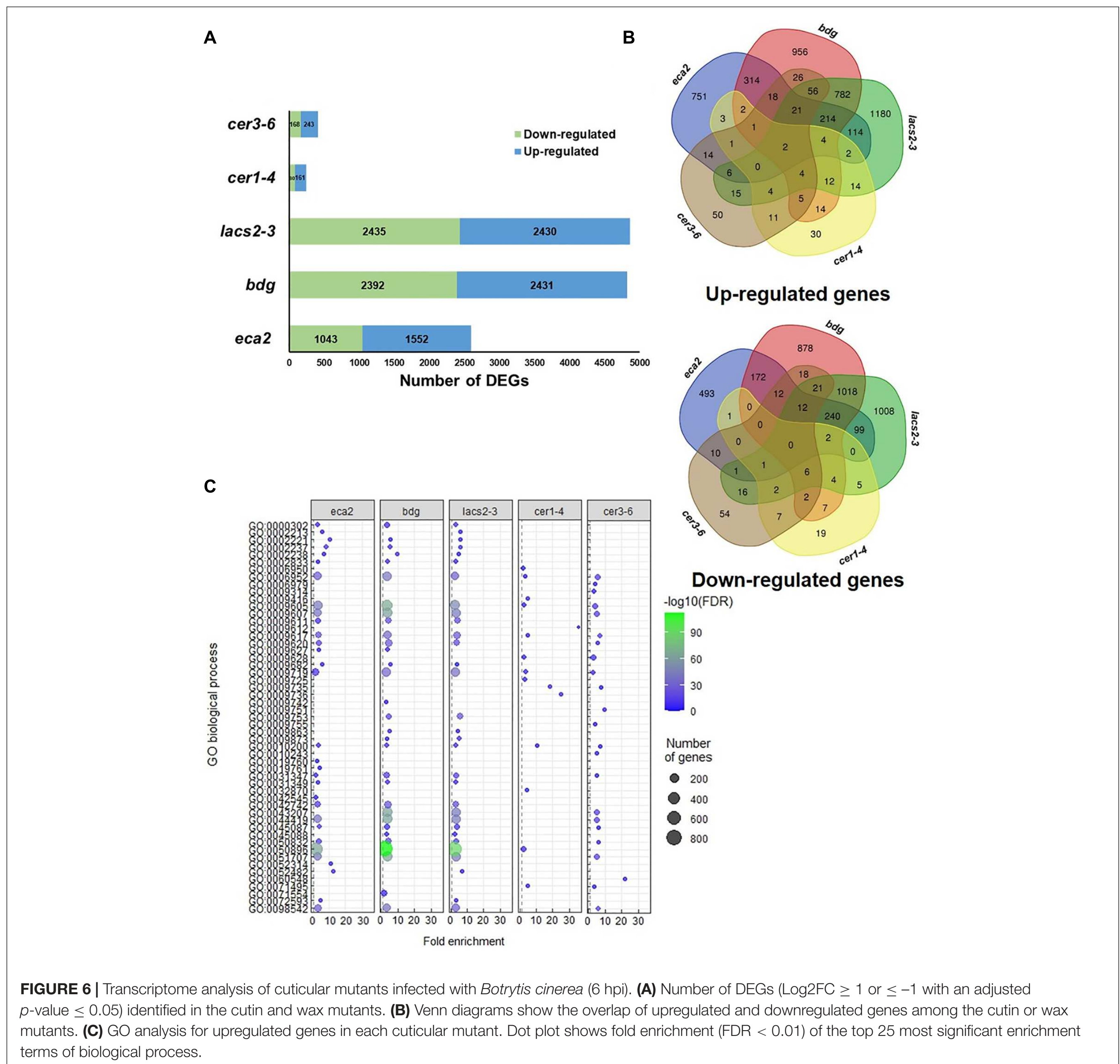

FIGURE 6 | Transcriptome analysis of cuticular mutants infected with *Botrytis cinerea* (6 hpi). **(A)** Number of DEGs (Log2FC ≥ 1 or ≤ −1 with an adjusted *p*-value ≤ 0.05) identified in the cutin and wax mutants. **(B)** Venn diagrams show the overlap of upregulated and downregulated genes among the cutin or wax mutants. **(C)** GO analysis for upregulated genes in each cuticular mutant. Dot plot shows fold enrichment (FDR < 0.01) of the top 25 most significant enrichment terms of biological process.

organisms (**Table 1**). Interestingly, among these DEGs, we found genes that are involved in cell wall remodelings, such as *AtPME17* (AT2G45220) and *AtPME41* (AT4G02330), and encoding pectin methylesterases (PMEs), which have been recently identified in response to *B. cinerea* (Lionetti et al., 2012; Bellincampi et al., 2014; Bethke et al., 2014, 2016). Likewise, two members of the APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) family, transcription factors *RAP2.6/ERF108* and *RAP2.6L/ERF113*, catalase *CAT3*, peroxidases *AtPRX71* [recently identified in resistance to *B. cinerea* (Lorrai et al., 2021)], and Sugar Transporter Protein *STP13,* and genes related to pattern-recognition receptors (PRRs), such as *RLK7*, *RLK5,* and *RLP30,* were identified as DEGs (**Figure 8A**). In order to confirm these expression patterns and to validate our RNA-seq analysis, we analyzed the expression of *AtPME17*, *AtPME41*, *RAP2.6/ERF108,* and *CAT3* by RT-qPCR (**Figure 8B**). As expected, we found that all these selected genes were induced 6 hpi in *eca2*, *bdg,* and *lacs2-3*, and that they were downregulated in *cer1-4* and *cer3-6* (**Figure 8B**).

DISCUSSION

Plants have developed sophisticated responses, such as effects of abiotic and biotic stimuli, to survive in a challenging environment. One of these mechanisms includes preformed

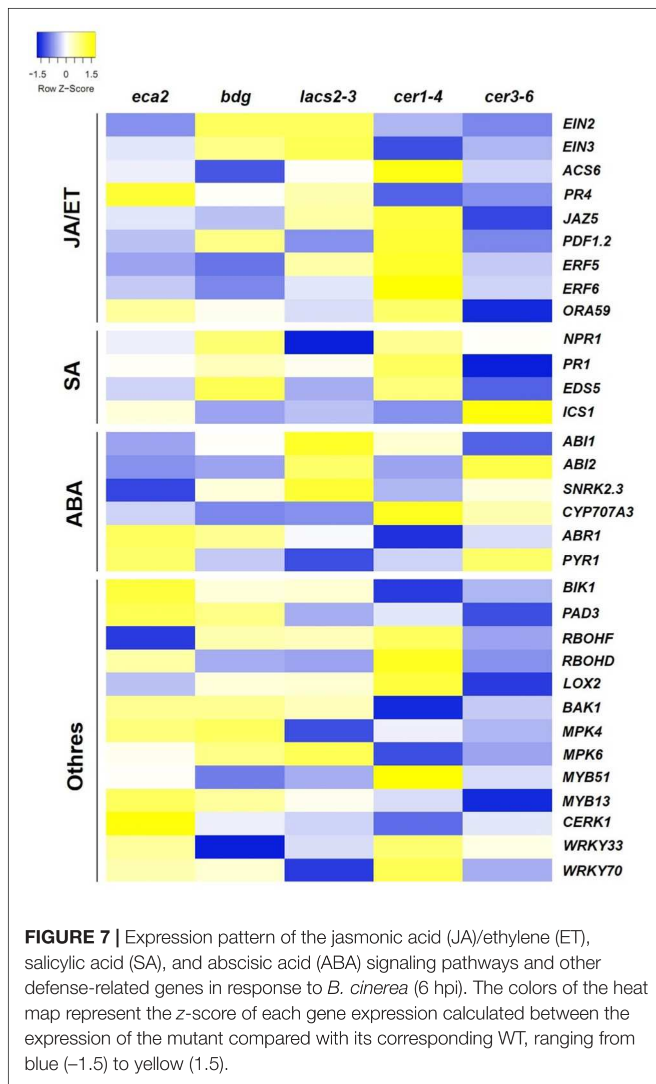

FIGURE 7 | Expression pattern of the jasmonic acid (JA)/ethylene (ET), salicylic acid (SA), and abscisic acid (ABA) signaling pathways and other defense-related genes in response to *B. cinerea* (6 hpi). The colors of the heat map represent the *z*-score of each gene expression calculated between the expression of the mutant compared with its corresponding WT, ranging from blue (–1.5) to yellow (1.5).

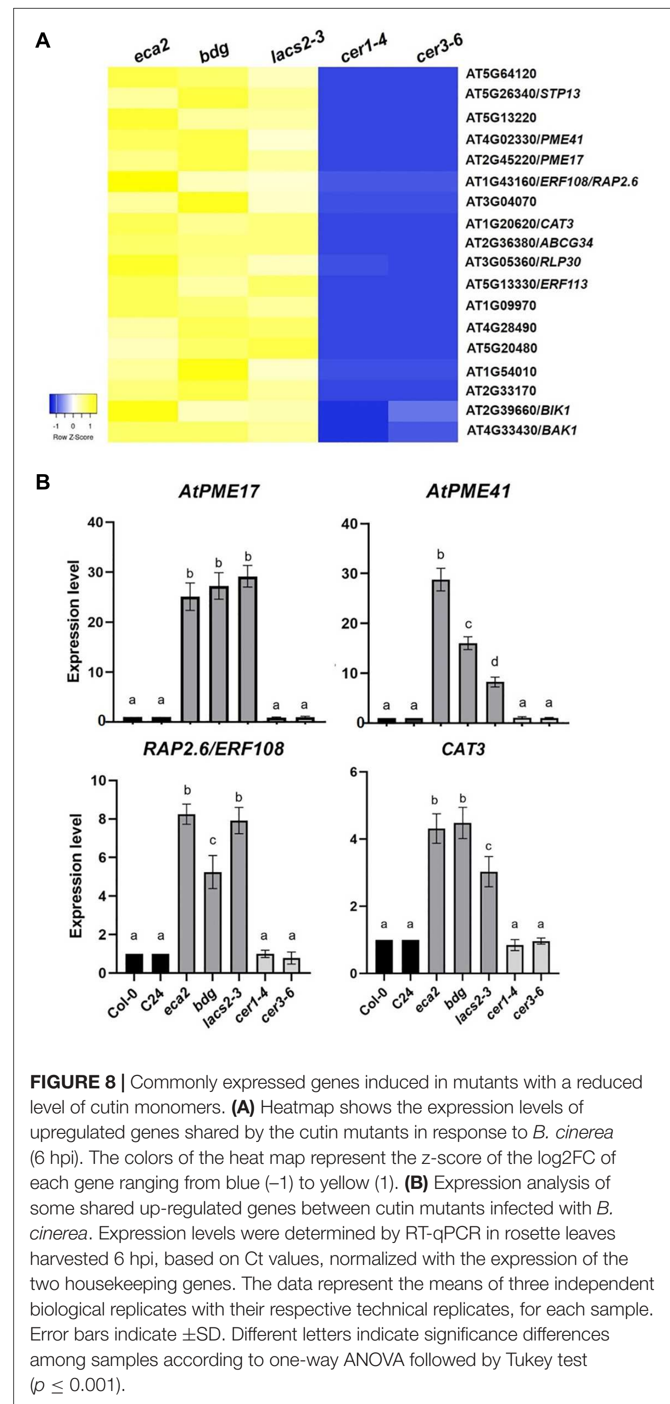

FIGURE 8 | Commonly expressed genes induced in mutants with a reduced level of cutin monomers. **(A)** Heatmap shows the expression levels of upregulated genes shared by the cutin mutants in response to *B. cinerea* (6 hpi). The colors of the heat map represent the z-score of the log2FC of each gene ranging from blue (–1) to yellow (1). **(B)** Expression analysis of some shared up-regulated genes between cutin mutants infected with *B. cinerea*. Expression levels were determined by RT-qPCR in rosette leaves harvested 6 hpi, based on Ct values, normalized with the expression of the two housekeeping genes. The data represent the means of three independent biological replicates with their respective technical replicates, for each sample. Error bars indicate ±SD. Different letters indicate significance differences among samples according to one-way ANOVA followed by Tukey test ($p \leq 0.001$).

physical barriers on the plant surface, such as the cuticle (Chassot et al., 2007; Serrano et al., 2014; Aragón et al., 2017; Bacete et al., 2018; Engelsdorf et al., 2018; Molina et al., 2021). The cuticle, mainly formed by cutin and waxes, also serves as a source of signaling molecules that coordinate the dialog between plants and microorganisms (Aragón et al., 2017; Ziv et al., 2018). In *A. thaliana* and *Solanum lycopersicum*, changes in permeability, associated with modifications in cutin composition, have been linked to resistance against the necrotrophic fungus *B. cinerea* (Bessire et al., 2007; Chassot et al., 2007; Isaacson et al., 2009; Voisin et al., 2009; Martin et al., 2017; Blanc et al., 2018). This resistance has been attributed to faster recognition of pathogens due to a permeable cuticle, accumulation of ROS, and induction of plant defense responses. However, plants with reduced content of waxes, which also have a modified cuticle structure, have not been characterized in detail with respect to this phenotype. In this study, we determined that the wax mutants have a more permeable cuticle but are as susceptible as the wild-type plants. This is in line with the fact that not all cuticle mutants show resistance to *B. cinerea*. For instance, the cuticle mutants *acp4* and *gl1* showed susceptibility to both bacterial pathogens *Pseudomonas syringae* and *B. cinerea* (Xia et al., 2009, 2010; Benikhlef et al., 2013; Lim et al., 2020). Similarly, *shn1*, with cutin monomer content altered, has a more permeable cuticle but is more susceptible to three necrotrophic fungal pathogens, *B. cinerea*, *S. sclerotiorum,* and *Alternaria brassicicola* (Sela et al., 2013; Buxdorf et al., 2014). These results suggest that, as expected, cuticle structural integrity is an important physical barrier against

TABLE 1 | GO enrichment analysis of Biological process of the 214 common upregulated genes in *eca2*, *bdg* and *lacs2-3* infected with *Botrytis cinerea* (genes are listed in **Supplementary Data 6**).

GO_ID	GO description	Gene number	Fold enrichment	*p-value*
GO:0002237	response to molecule of bacterial origin	5	18.3	3.76E-02
GO:0045087	innate immune response	9	8.71	4.46E-03
GO:0002376	immune system process	10	6.82	9.56E-03
GO:0010038	response to metal ion	18	6.28	3.62E-06
GO:0009620	response to fungus	12	5.15	1.65E-02
GO:0033993	response to lipid	24	4.44	4.83E-06
GO:0010035	response to inorganic substance	27	4.43	4.33E-07
GO:0098542	defense response to other organism	23	4.28	2.10E-05
GO:0043207	response to external biotic stimulus	29	3.97	1.01E-06
GO:0051707	response to other organism	29	3.97	1.01E-06
GO:0009607	response to biotic stimulus	29	3.97	1.03E-06
GO:0044419	biological process involved in interspecies interaction between organisms	29	3.88	1.70E-06
GO:0006952	defense response	24	3.79	9.29E-05
GO:0009605	response to external stimulus	35	3.47	4.23E-07
GO:0048583	regulation of response to stimulus	17	3.42	4.03E-02
GO:0042221	response to chemical	61	3.41	1.19E-14
GO:0019752	carboxylic acid metabolic process	19	3.38	1.43E-02
GO:1901700	response to oxygen-containing compound	34	3.37	1.72E-06
GO:0006082	organic acid metabolic process	22	3.36	2.72E-03
GO:0043436	oxoacid metabolic process	21	3.33	5.46E-03
GO:0009725	response to hormone	27	3.25	2.58E-04
GO:0009719	response to endogenous stimulus	27	3.18	4.12E-04
GO:0010033	response to organic substance	35	3.07	1.02E-05
GO:0044281	small molecule metabolic process	29	2.92	7.32E-04
GO:0006950	response to stress	59	2.84	1.60E-10
GO:0050896	response to stimulus	91	2.47	1.89E-15
GO:0009987	cellular process	126	1.59	1.69E-08
GO:0008152	metabolic process	84	1.51	3.41E-02

multiple pathogens (such as *B. cinerea*), and that mutants *sma4, lcr*, *bdg, lacs2,* and *eca2,* which possess a low content of cutin, a permeable cuticle, and are resistant to *B. cinerea,* are exceptions to the rule. Additionally, our results indicate that faster recognition of the pathogen by changing cuticle permeability is not enough to induce plant innate immunity responses and resistance to *B. cinerea,* as previously hypothesized (Bessire et al., 2007; Serrano et al., 2014). Nevertheless, these cutin mutants can be used as models to investigate the molecular mechanisms behind the successful induction of defense responses against this agronomically important pathogen.

Plant development and responses to the environment are induced and regulated by signaling networks of "*trio signaling*" messengers: ROS, electrical signals, and calcium (Choi W.G. et al., 2017). In particular, ROS are induced by activating various oxidases and peroxidases in response to abiotic and biotic conditions (Torres and Dangl, 2005; O'Brien et al., 2012; Baxter et al., 2014; Saini et al., 2018). During host-pathogen interactions, the production of ROS is important for plants and for necrotrophic fungi, such as *B. cinerea* and *S. sclerotiorum*. For instance, one of the earliest defense responses to a pathogen attack is the so-called "oxidative burst," the production of ROS at the site of invasion. A plant releases high amounts of reactive oxygen species (ROS) to counteract the pathogen. On the other hand, *Botrytis cinerea* also takes advantage of this plant defense to kill host cells before they are invaded by hyphae and is able to cope with external oxidative stress in order to survive in the necrotic tissue (Siegmund and Viefhues, 2016). We have described a direct relationship between the accumulation of ROS and resistance to *B. cinerea* after mechanical stimulus or by triggering defense responses through elicitors (Benikhlef et al., 2013; Narváez-Barragán et al., 2020; Batista-Oliveira et al., 2021). Additionally, the resistance against this pathogen observed in the cutin mutants has been also linked to increased levels of ROS under non-infected conditions (reviewed in Serrano et al., 2014). Here, we show that under non-challenged conditions, although all the mutants accumulated more ROS than the WT plants, the cutin mutants *eca2*, *bdg,* and *lacs2-3* have higher levels of superoxide and hydrogen peroxide than the wax mutants *cer1-4* and *cer3-6* (**Figure 3**). Interestingly, at 6 hpi, all the cuticular mutants have higher levels of superoxide and hydrogen peroxide than the WT plants (**Supplementary Figure 1**). Despite the *B. cinerea*-induced ROS burst observed in the wax mutants, they showed susceptibility. Two possibilities can be explored to explain this phenotype based on ROS accumulation. First, since a moderate level of ROS is detected in the susceptible mutants *cer1-4* and *cer3-6*, the level of ROS during the initial interaction with the pathogen could be important in triggering resistance against this pathogen. In agreement with this, it has been described that the inhibition of the size of lesions caused by *B. cinerea* is directly proportional to the level of ROS induced by soft mechanical stress (Benikhlef et al., 2013). On the other hand, during biotic interactions, ROS have been characterized to participate in multiple processes, such as reinforcement of the cell wall, regulation of hormone-induced signaling pathways, and triggering of hypersensitive response (Lehmann et al., 2015). Since we observed an increase of ROS in the wax mutants only after the interaction with the pathogen, it is possible that timing, not only the activation of the oxidative burst, is important to efficiently build up a defense response. Either way, our results suggest that besides ROS-dependent resistance, it is probable

that other(s) mechanism(s) may be involved to trigger the plant response and, therefore, induce resistance against *B. cinerea*.

To further characterize the molecular mechanisms underlying the resistance or susceptibility in the cuticular mutants, we performed a transcriptomic analysis on the non-infected plants. Up-regulated genes identified in the cutin mutants were classified into the response to biotic stimulus, while GO terms in *cer1-4* and *cer3-6* were mainly associated with response to abiotic stimulus (**Figure 5**), suggesting that the identified DEGs in cutin mutants could be part of a primed defense response mechanism. These results are in line with previous reports, where a defense priming mechanism has been observed in plants treated with cutin monomers, as well as in other cuticular mutants, leading to expression of a suite of faster and stronger defense responses upon challenge with *B. cinerea* (Schweizer et al., 1996; Fauth et al., 1998; Bessire et al., 2007; Chassot et al., 2007; Conrath, 2011; Conrath et al., 2015; Mauch-Mani et al., 2017). Notably, our data also identified genes related to PRRs, highlighting the leucine-rich repeat receptor kinase (LRR-RK) (**Figure 4**). Previous studies have reported the importance of PRRs in the priming state. The plant receptor FLS2 and its co-receptor BAK1 were associated with the enhanced responsiveness of *Arabidopsis* plants to the bacterial flagellin peptide flg22 (Tateda et al., 2014), as well as the malectin-like LRR receptor-like kinase IOS1, which is associated with FLS2 and the bacterial receptor EF-Tu (Yeh et al., 2016). Taken together, our results suggest that before the interaction with the pathogen occurs, the cutin mutants are in a priming state compared with the wax mutants or WT plants and that once the infection takes place, they might respond in a faster and more efficient manner, stopping the infection.

Previous reports have characterized secondary responses transcriptionally induced during the interaction with *B. cinerea*. Among these defense responses, SA-, ET-, ABA- and JA-related pathways are induced (AbuQamar et al., 2006; Mengiste, 2012; Windram et al., 2012). To determine if a similar set of genes was present in the cutin and wax mutants, we performed a transcriptome analysis at 6 hpi. In *cer1-4* and *cer3-6* the defense responses are not induced, and most of the DEGs were classified into the response to abiotic stimulus, while in the cutin mutants, enrichment of defense-related genes was identified (**Figure 6**). However, in *eca2*, *bdg,* and *lacs2-3,* a similar profile of these defense marker genes, which could explain the resistance phenotype for all these mutants based on these hormone-induced responses, was not observed (**Figure 7**). From this set of genes, only two, involved in the early stages of recognition of pathogen, were identified to be induced in all the cutin mutants: the LRR receptor-like protein kinase (*BAK1*) and its interactor *Botrytis-induced kinase 1* (*BIK1*). This result is in accordance with previous reports showing that the *Arabidopsis* mutant *sma4* with a defective cuticle displayed increased resistance to *B. cinerea*, and that this process was

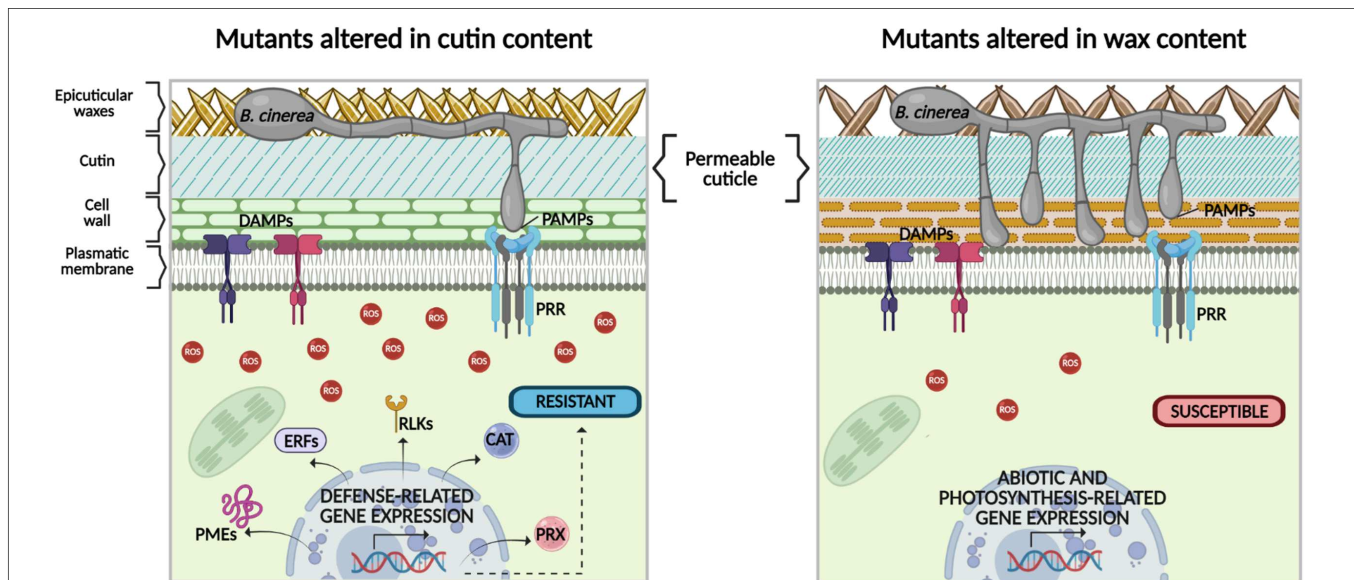

FIGURE 9 | Hypothetical model of the role of cuticular components cutin and wax in differential response to the necrotrophic fungus *B. cinerea*. In previous studies, a direct relationship between increased permeability in cutin mutants and resistance to *B. cinerea* has been proposed. However, our data show that despite both cuticular mutants having enhanced cuticular permeability, compared with the WT plants, only mutants with altered cutin content show resistance to *B. cinerea*. This phenotype could be explained as follows: (1) in the cutin mutants, once plant plasma membrane-localized receptors recognize pathogen-/damage-associated molecular patterns (PAMPs/DAMPs) a more intense ROS production is observed, compared with mutants with altered wax content. (2) During cutin mutant-*B. cinerea* interaction, defense-related signaling pathways are triggered, which are different from canonical immune ones, such as expression genes encoding catalases (*CAT3*) and peroxidases (*PRX*), as well as LRR-receptor-like kinases (*RLK5, RLK7, RLP30*), transcription factors (*ERF108* and *ERF113*), and genes involved in the remodeling of the cell wall, such as *PMEs*. (3) In contrast, according to our data, in the mutants with altered wax content, ROS production is not enough to effectively prevent *B. cinerea* colonization. In these mutants, other secondary signals might be activated to control the expression of genes related to abiotic stimuli, instead of the expression of defense-related genes (the image was designed using bioRENDER application, https://biorender.com/).

independent of the JA and ET signaling pathways (Tang et al., 2007; Wang et al., 2020). Similarly, in CUTE plants, resistance to *B. cinerea* was not found to correlate with the induction of genes associated with the SA, ET, or JA signaling pathways (Chassot et al., 2007). In sum, these results suggest that the resistance to *B. cinerea* observed in *eca2*, *bdg,* and *lacs2-3* might not only be driven by the induction of canonical defense responses, previously identified to be important in stopping the infection.

Since the stronger difference between cutin and wax mutants was resistance and susceptibility to *B. cinerea,* respectively, we hypothesized that a set of genes should be induced only in *eca2*, *bdg,* and *lacs2-3* but repressed in *cer1-4* and *cer3-6* (**Supplementary Data 7**). Among these DEGs, we identified genes previously characterized to be involved in ROS regulation and cell wall biosynthesis, and are discussed next. The peroxidase (At*PRX71*) and catalases (*CAT3* andAT4G37530) genes were identified as DEGs in the cutin mutants. These genes have been described as part of ROS-scavenging systems to maintain ROS homeostasis in different compartments of the cell, and could, thus, restrict the ROS-dependent damage or finely coordinate the ROS-dependent signal transduction in the presence of a pathogen (Torres et al., 2006; O'Brien et al., 2012). However, necrotrophic pathogens also produce ROS to kill host cells triggering a hypersensitive reaction (HR), thereby facilitating the infection (Govrin and Levine, 2000; Torres et al., 2006; van Kan, 2006). Based on this evidence, it is possible that the expression of these scavengers might help either the plant to inhibit the proper ROS-induced infection process of *B. cinerea*, or regulate ROS-dependent plant defense responses. Clearly, this hypothesis should be tested in future studies.

Additionally, we identified the induction of pectin methylesterases (PMEs) *AtPME41* and *AtPME17* (**Figure 8**) in the cutin mutants in response to *B. cinerea*. Importantly, these genes have not been reported before as a part of the defense genes against *B. cinerea* in any cuticular mutants. Our data are in accordance with previous results showing that plants activate a local and strong PME activity in response to pathogens with different lifestyles (Lionetti et al., 2012; Bethke et al., 2014; Lionetti, 2015). Once the pathogen overcomes the cuticle, the pectin matrix, which is the major fraction of the cell wall of dicots and non-aminaceous monocots, is the next target for fungal necrotrophs (van Kan, 2006; Laluk and Mengiste, 2010; Lionetti et al., 2012; Bellincampi et al., 2014). PME activity was proposed to be involved in the release and perception of defense of endogenous signals with elicitor activities from cellular components during infection, such as oligogalacturonides (OGs) considered as DAMPs (Lionetti et al., 2012; Ferrari et al., 2013; De Lorenzo et al., 2019). *AtPME41* synthesizes a member of PMEs that has an important role in activating the immune response when *Arabidopsis* is challenged with the necrotroph *A. brassicicola* (Lionetti et al., 2012; Bethke et al., 2014). Additionally, Del Corpo et al. demonstrated the functional role of AtPME17 in triggering PME activity by the JA/ET-dependent pathway and in resistance against pectinolytic necrotrophic fungi, such as *B. cinerea* (Del Corpo et al., 2020).

Finally, this study illustrates that *B. cinerea* resistance in the cutin mutants *eca2*, *bdg,* and *lacs2-3,* compared with *cer1-4* and *cer3-6*, clearly consists of a multitude of signaling events, from pre-activated defense or priming as initial resistance, production of ROS, and increased expression of non-canonical defense-related genes (**Figure 9**).

CONCLUSION

We have shown that while *cer1-4* and *cer3-6* have altered cuticle permeability, they present a susceptible phenotype, suggesting that the faster recognition of the pathogenic fungus *B. cinerea* is not enough to induce plant innate immune responses and resistance as previously hypothesized. In the cutin mutants *eca2*, *bdg,* and *lacs2-3* responding to *B. cinerea,* a profile of previously characterized defense-response gene markers was not observed, suggesting that mutants with resistant phenotypes can activate other defense pathways, different from these canonical immune ones. Nevertheless, we identified the induction of genes involved in cell wall remodeling in the cutin mutants, which have not been previously reported as part of the defense genes against *B. cinerea* in any cuticular mutants. Based on these results, our study can be used as a starting point to understand the molecular basis involved in early defense mechanisms related to cuticular components against this agronomically important necrotrophic pathogen.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject; PRJNA761130.

AUTHOR CONTRIBUTIONS

WA, DF, and MS conceived and designed the experiments. WA, DF, NA-B, and MT performed the experiments. WA, DF, and MS wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.738949/full#supplementary-material

Supplementary Figure 1 | Reactive oxygen species (ROS) production at 6 hpi with *Botrytis cinerea* in leaves from 4-week-old plants of *Arabidopsis thaliana* cuticular mutants and wild-type (WT) Columbia-0 (Col-0) and C24 plants. Fluorescence after DCF-DA staining was observed on leaves by using epifluorescence microscopy. 3, 3′-Diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) were used to detect the accumulation of hydrogen peroxide (H_2O_2) and superoxide (O_2^-), respectively. The experiment was carried out six times, with similar results ($n = 6 \pm$ SD). Scale bar = 100 μm. Different lowercase letter columns indicate significant differences, according to one-way analysis of variance (ANOVA) (p-value < 0.001) followed by Tukey's test. Representative pictures are shown.

Supplementary Figure 2 | The heat map shows the expression pattern in non-infected cutin and wax mutants, as well as the WT plants of the jasmonic acid (JA)/ethylene (ET), salicylic acid (SA), and abscisic acid (ABA) signaling pathways, and other defense-related genes selected from the literature. The colors of the heat map represent the z-score of each gene ranging from blue (−2) to yellow (2).

Supplementary Table 1 | Cuticular wax and cutin composition of rosette leaves of Arabidopsis Col-0 (wt), *cer1-4*, *cer3-6* (yre), *bdg*, *lacs2-3*, C24 (wt), and *eca2*.

Supplementary Table 2 | Summary of bioinformatics data and mapped reads of *Arabidopsis thaliana* cutin and wax mutants in the RNA-seq libraries in non-infected and infected with *Botrytis cinerea* (6 hpi).

Supplementary Data 1 | List of significantly differentially expressed genes (DEGs) in non-infected cutin and wax mutants, related to **Figure 4A**. List of defense-related genes abundant in the non-infected mutants and wild-type (WT) plants, related to **Supplementary Figure 2**.

Supplementary Data 2 | List of up- and down-regulated genes shared by non-infected cutin or wax mutants, related to **Figure 4B**.

Supplementary Data 3 | Gene Ontology (GO) term enrichment of up- and down-regulated genes shared by non-infected cutin or wax mutants, related to **Figure 4B**.

Supplementary Data 4 | GO term enrichment of up- and downregulated genes in each non-infected cutin or wax mutant, related to **Figure 5**.

Supplementary Data 5 | List of significantly DEGs in cutin and wax mutants infected with *B. cinerea* (6 hpi), related to **Figure 6A**. List of defense-related genes abundant in the infected mutants and WT plants, related to **Figure 7**.

Supplementary Data 6 | List of up- and downregulated genes shared by cutin or wax mutants infected with *B. cinerea* (6 hpi), related to **Figure 6B**.

Supplementary Data 7 | GO term enrichment of up- and downregulated genes shared by infected cutin or wax mutants, related to **Figure 6C**.

Supplementary Data 8 | GO term enrichment of up- and downregulated genes shared by infected cutin or wax mutants, related to **Figure 6B**.

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CAPÍTULO IV.
Resultados no publicados.
Identificación genética de *eca2*.

En este capítulo se describen los principales resultados no publicados, pero que también fueron producto de este trabajo doctoral. Simultáneamente al estudio de cómo la composición de la cutícula contribuye a los mecanismos de defensa contra el hongo *B. cinerea*, se realizaron esfuerzos para responder parcialmente a la pregunta de investigación: ¿Cuál es el gen afectado o responsable del fenotipo de la mutante *eca2*, afectada en el contenido de cutina y de ceras de su cutícula foliar?, a través del análisis de las secuencias de gADN de una población de mapeo.

IV.1 Introducción.

Durante el transcurso del tiempo, se han implementado diferentes estrategias para identificar y caracterizar la función de los genes en las plantas y en otros organismos, entre ellas están la genética directa (del inglés *forward genetic*) y genética reversa (del inglés *reverse genetic*). La genética reversa, parte de un gen o familia de genes es mutado, ya sea en la secuencia codificante o en el nivel y patrón de expresión, para predecir su función a través del estudio de los efectos de su alteración. En la genética directa se identifican y seleccionan individuos de una población mutante que posean características o rasgos observables (fenotipo), para después identificar el gen o genes afectados (genotipo) en dichos individuos, responsables de esas características. (Alonso y Ecker, 2006).

El proceso de escrutinio de la genética directa comienza con una mutagénesis al azar, para introducir variaciones genéticas que ocasionalmente conducen a fenotipos de interés. Posteriormente, se realiza un mapeo genético o físico para encontrar el *locus* genético mutado y después determinar la secuencia del gen responsable (Lukowitz et al., 2000). En las plantas, la identificación de genes que contribuyen a las variaciones en el fenotipo tiene muchas implicaciones, no solo para entender procesos biológicos fundamentales, sino también para aplicar ese conocimiento en el mejoramiento de los cultivos.

IV.1.1 La genética directa es facilitada por tecnologías de secuenciación.

La última década ha sido testigo de la revolución en la secuenciación del ADN, desde la aparición de la tecnología de Sanger hasta las plataformas como Illumina y Nanopore, siendo estas últimas parte de las tecnologías de secuenciación de nueva generación, **NGS** (del inglés, *Next Generation Sequencing*). El crecimiento y el rendimiento de estas, no solo han influido en los proyectos de secuenciación de genomas completos de plantas, sino también en los proyectos de re-secuenciación y en la identificación de variaciones naturales entre accesiones y/o ecotipos. El número de genomas de diferentes especies de plantas que se han secuenciado está creciendo a gran velocidad, a medida que el costo por par de bases secuenciadas se está reduciendo. Proyectos como el de 1,001 genomas de Arabidopsis (http://1001genomes.org), donde la

secuenciación de múltiples ecotipos, ha dado lugar a un conjunto de datos de variaciones naturales que tienen implicaciones directas en estudios genómicos, tales como perfiles de todo el genoma, **WSP** (del inglés, *Whole Genome Profiling*) y estudios de asociación de todo el genoma, **GWA** (del inglés, *Genome Wide Association*) (Schneeberger y Weigel, 2011; Schneeberger, 2014).

Durante varias décadas, la genética directa ha sido una técnica poderosa para identificar genes y mutaciones que se asocian a fenotipos de interés. En casi todos los sistemas biológicos, el escrutinio en la genética directa, sigue los mismos principios básicos. Los organismos con un fondo genético bien definido, se mutagenizan usando agentes que dañan el ADN, tales como la irradiación o mutágenos químicos, los cuales introducen mutaciones al azar. Entre estos últimos, el más utilizado en Arabidopsis es el etil-metano-sulfonato (EMS). Una vez que se obtiene la población de mutantes, es sometida al escrutinio para seleccionar aquellas plantas que muestren el fenotipo de interés. La búsqueda de la mutación genética responsable del fenotipo, es un proceso de varios pasos, donde uno de ellos es el mapeo genético, para localizar una región cromosómica amplia que alberga la mutación (intervalo de mapeo), seguido de una búsqueda dirigida para mutaciones candidatas dentro de esta región (Schneeberger, 2014). La validación de que un gen candidato mutado es responsable del fenotipo, a menudo implica la confirmación, a través del genotipado de individuos mutantes e individuos de tipo silvestre en el *locus* putativo; así como también, a través de enfoques funcionales, como por ejemplo que un alelo de tipo silvestre de ese gen, pueda complementar el alelo afectado en la mutante y rescatar el fenotipo, toda vez que este debe ser idéntico al de su planta de tipo silvestre (Page y Grossniklaus, 2002; Schneeberger, 2014).

En los últimos años, el mapeo genético ha evolucionado considerablemente debido a diversos desarrollos tecnológicos y metodológicos. En un escenario típico, se aísla un mutante con un fenotipo de interés y se utiliza un esquema de cruzas bien definido para generar una población de mapeo recombinante, que contiene tanto individuos mutantes, como individuos de tipo silvestre. Los individuos recombinantes que muestran el fenotipo mutante, son portadores de la mutación, aunque de ubicación

desconocida. Debido a la recombinación poco frecuente, entre la mutación que causa el fenotipo y los marcadores genéticos cercanos, los alelos de estos marcadores genéticamente vinculados, co-segregarán con la mutación que causa el fenotipo, mientras que los marcadores alelos no vinculados, mostrarán segregación al azar entre los individuos mutantes y de tipo silvestre. Por lo tanto, la selección de mutantes recombinantes, da como resultado una distribución alélica distinta, tanto de la mutación que causa el fenotipo, como de los marcadores estrechamente vinculados (Schneeberger, 2014). Los análisis de genotipado, pueden detectar la sobre-representación de alelos, que se derivan del fondo mutante y dirigen los análisis posteriores hacia la región que alberga la mutación que causa el fenotipo. Por lo anterior, el análisis de frecuencias alélicas en poblaciones recombinantes se pueden usar para asociar el fenotipo a una región genómica, posibilitando así el mapeo genético a través de frecuencias alélicas en un conjunto de ADN (Schneeberger y Weigel, 2011; Schneeberger, 2014).

La resolución del mapeo genético depende entonces, entre otros factores, de encontrar una región de baja recombinación en el genoma de la población recombinante de mapeo, y así encontrar polimorfismos asociados a la mutación. Con el lanzamiento de los primeros genomas de referencia de plantas y conjuntos de marcadores moleculares, estuvieron disponibles nuevos métodos de genotipado. Debido a la revolución en las tecnologías de secuenciación del ADN, ha emergido el mapeo por secuenciación, el cual combina el mapeo genético con la secuenciación del genoma completo (mapeo físico) para acelerar la identificación de la mutación que causa el fenotipo, a través del uso de tecnologías de secuenciación de nueva generación. Una de las ventajas de este tipo de enfoques, es la identificación de marcadores genéticos y la cuantificación de frecuencias de alelos locales en un conjunto de muestras de ADN de una población de mapeo, y la revelación de la secuencia de nucleótidos que permite simultáneamente el mapeo y la identificación de la mutación que causa el fenotipo (James et al., 2013; Schneeberger, 2014).

IV.1.2 SHOREmap, una herramienta en la genética directa.

En la planta modelo Arabidopsis, el método más utilizado para identificar los cambios inducidos por un mutágeno de uso común como el EMS (de C por T o G por A), ha sido una estrategia basada en la cartografía y en el posicionamiento clonal. Para llevar a cabo esta estrategia de forma rutinaria, se parte de cruzar una mutante de interés con una planta de tipo silvestre de diferente accesión o ecotipo, y utilizar los polimorfismos propios de cada ecotipo, asociándolos a un fenotipo través del análisis de ligamiento genético. En el caso de mutantes recesivas, el siguiente paso es el análisis de cada individuo que presenta el fenotipo de interés entre la población recombinante, utilizando diversos marcadores para detectar cada polimorfismo (Qu y Qin, 2014). La identificación de la variación del gen que causa el fenotipo, suele comenzar con un mapeo de todo el genoma (mapeo grueso), que acota la región cromosómica que contiene la mutación, seguido de un mapeo adicional (mapeo fino), para después identificar la mutación que causa el fenotipo, mediante la secuenciación de los genes candidatos y, finalmente, la validación de los genes por la complementación genética. En general, este método requiere mucho tiempo y trabajo, y se hace más difícil cuando hay múltiples *loci* ligados al fenotipo. Algunos informes recientes, han demostrado que las mutaciones inducidas por EMS pueden identificarse con menos trabajo mediante tecnologías de secuenciación, cuando se generan mutantes en el fondo genético de referencia Columbia (Col-0) de Arabidopsis, cuya secuencia genómica es bien conocida (Ossowski et al., 2008; Schneeberger y Weigel, 2011).

Recientemente, han surgido procedimientos que tienen como objetivo simplificar el proceso tradicional de mapeo. Entre ellos se encuentra el método de SHOREmap (Schneeberger et al., 2009), el cual se basa en la re-secuenciación del genoma completo de mutantes recombinantes y los principios del análisis de un grupo de plantas segregantes, reduciendo así la carga de trabajo real, desde el establecimiento de las poblaciones de mapeo, hasta la validación de genes candidatos. Las primeras demostraciones de este método se realizaron en Arabidopsis, donde las mutaciones derivadas del EMS se mapearon utilizando un grupo de plantas segregantes, a partir de la cruza de la mutante en el fondo genético del ecotipo Col-0, con una planta tipo silvestre Landsberg erecta (Ler). En este método, las regiones que son escasas de **SNP** o

snips como suele pronunciarse (del inglés, *Single Nucleotide Polymorphisms*), derivadas del ecotipo Ler, se toman como aquellas que albergan la mutación, y se analizan para determinar la frecuencia de **SNP** inducidas por EMS. Es decir, se puede estimar la frecuencia alélica (**FA**) provenientes de los alelos parentales en todo el genoma de las plantas recombinantes, para identificar el sesgo local que fue introducido por la selección del fenotipo mutante, y revelar la región que alberga la mutación que causa el fenotipo (Schneeberger et al., 2009). La utilidad del flujo de trabajo del método de SHOREmap, se ha extendido para incluir el mapeo de mutaciones no relacionadas con el mutágeno químico EMS en plantas no modelo (Guo et al., 2012). Métodos similares se han desarrollado, tanto para Arabidopsis como para otras especies vegetales (Austin et al., 2011; Wachsman et al., 2017).

El mapeo por secuenciación, como está implementado en la herramienta de SHOREmap, se basa en una lista de marcadores polimórficos entre los alelos parentales. Puede utilizar diferentes tipos de poblaciones de mapeo segregante, tanto de una derivada de la cruza de la mutante con un ecotipo divergente (**OC**, del inglés *outcross*), como aquella derivada de la retrocruza de la mutante con su parental (**BC**, del inglés *backcross*) (Sun y Schneeberger, 2015). En la población derivada de una cruza tipo **OC**, se introduce gran número de polimorfismos naturales en el genoma de las plantas recombinantes, los cuales, junto con los cambios inducidos por el mutágeno, sirven como marcadores que serán utilizados para estimar la frecuencia alélica y estimar la región cromosómica de la mutación (Galvão et al., 2012; Allen et al., 2013; James et al., 2013). Mientras que en la población de mapeo generada por una retrocruza, los cambios inducidos por el mutágeno se pueden identificar a partir, de los datos de secuenciación del genoma completo de las mutantes recombinantes, ya que van a segregar como polimorfismos naturales, que finalmente son utilizarlos para el mapeo (Hartwig et al., 2012; Allen et al., 2013).

IV.1.3 Identificación y caracterización de la mutante *eca2*.

Con el objetivo de identificar mutantes que modificaran el patrón de expresión del gen de respuesta temprana a elicitores *ATL2*, se realizó un escrutinio genético utilizando una línea transgénica, en el fondo genético del ecotipo C24, que contiene la región

promotora del gen *ATL2* fusionada al gen reportero *GUS* (p*ATL2::GUS*). De esta manera, se identificaron 5 mutantes que expresaron constitutivamente el gen *ATL2*, que se denominaron ***eca*** (*expresión constitutiva de ATL2*). Por medio de una caracterización preliminar de estas mutantes, se determinó que todas ellas son recesivas y están afectadas en genes diferentes (Serrano y Guzmán, 2004). Asimismo, se observó que las mutaciones modificaron no solo la expresión del gen *ATL2*, sino de otros genes de defensa relacionados con las respuesta tempranas y tardías (Serrano y Guzmán, 2004). Posteriormente, con el objetivo de determinar la posible participación de estas mutantes en la regulación de las interacciones bióticas, se confrontaron con diferentes patógenos biótrofos y necrótrofos. De este estudio, se determinó que sólo la mutante ***eca2*** presenta una resistencia a la bacteria *P. syringae* y al hongo *B. cinerea*. Adicionalmente, se determinó que ***eca2*** presentó una mayor permeabilidad cuticular, acumulación de ROS y una activación temprana de los genes de defensa. Cabe resaltar que al realizar un análisis bioquímico de la cutícula foliar de ***eca2***, se determinó que presenta un contenido reducido tanto de ceras, como de cutina, siendo con ello la primera mutante conocida en tener el contenido disminuido de ambos constituyentes cuticulares (Blanc et al., 2018). Sin embargo, a la fecha la identidad genética del gen *ECA2* es aún desconocida, por ello, uno de los objetivos de este trabajo consistió en esfuerzos para su identificación por medio de las técnicas de secuenciación de nueva generación.

IV.2 Materiales y Métodos.

IV.2.1 Generación de población de mapeo de la mutante *eca2*.

Es importante mencionar que previamente se realizó la cruza de la mutante ***eca2*** (en el fondo genético del ecotipo C24), con su línea parental tipo silvestre wt (p*ATL2*::GUS); asimismo se hizo el análisis de segregación (Aviles-Baltazar, 2018). La población de la generación F1 resultante de dicha cruza presentaba un fenotipo tipo silvestre (dominante) (**Figura 10**). A partir de la autofertilización de las plantas F1, se originó la población F2 segregante. Mediante el análisis del patrón de expresión del gen reportero, se confirmó que la mutación en ***eca2*** es recesiva, con una segregación Mendeliana 3:1 de acuerdo con la prueba de ji-cuadrada (χ^2) (**Tabla 1**). Posteriormente, se recuperaron las semillas y se realizó el escrutinio de la población de mapeo con diferentes poblaciones de cruzas. Para ello, se sembraron semillas de distintas poblaciones F2 segregante en condiciones *in vitro*, en placas con medio Murashige y Skoog (MS) 0.5X, a pH 5.7 (Murashige y Skoog, 1962). Las semillas fueron previamente esterilizadas con etanol al 70% y etanol absoluto, y estratificadas a 4 °C durante 48 h. Las cajas se mantuvieron a 22°C con un fotoperiodo de 16h/8h luz/oscuridad. A los 10 días, se tomó 1 hoja de cada una de las plantas de la población F2 de cada cruza evaluada y se les aplicó la tinción histoquímica de GUS (β-glucoronidasa) (Jefferson et al., 1986) para observar la expresión del promotor del gen *ATL2,* como criterio de selección para la población de mapeo de la retrocruza.

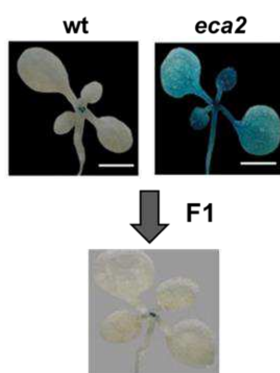

Figura 10. Fenotipo de la población F1 resultante de la cruza entre *eca2* X wt. Imagen adaptada de Aviles-Baltazar, (2018).

Tabla 1. Análisis genético de la mutante *eca2* en plantas adultas.

			Patrón de expresión		
Cruza (hembra X macho)	Generación	No. de plantas	wt	*eca2*	χ^2
ECA2/ECA2 X eca2/eca2	F1	15	15	0	
ECA2/ECA2 X eca2/eca2	F2	75	58	17	0.217; $P < 0.05$

χ^2 fue calculado para un radio esperado de 3:1, wt/mutante.

IV.2.2 Mapeo por secuenciación de la población F2 y del parental de *eca2*.

La población de mapeo derivada de la retrocruza consistió en hacer un conjunto de las plántulas segregantes F2 (población **BCF2**) que mostraron el fenotipo de interés de *eca2*. De manera similar, se recuperó un conjunto de plantas del parental tipo silvestre, con el objetivo de tener la secuencia como control en el análisis mediante la herramienta SHOREmap. Posteriormente, se realizó la extracción de ADN genómico (gADN) de cada uno de los conjuntos de plantas con el método del CTAB 2X (Edwards et al., 1991). La concentración y pureza de las muestras se evaluó cualitativamente, mediante un gel de agarosa, y de forma cuantitativa, usando el Nanodrop (Thermo Fischer Scientific) y el kit fluorométrico QUBIT (Thermo Fischer Scientific).

IV.2.3 Análisis de las secuencias mediante la herramienta SHOREmap.

El ADN genómico que se extrajo de las plantas seleccionadas se envió para la secuenciación del genoma a la empresa Beijing Genomics Institute (BGI) Americas (https://www.bgi.com/us), para realizar un WGS mediante la tecnología DNBSeq™. La secuenciación generó el formato de lecturas pareadas (*paired-end*) de 150 nucleótidos de longitud (2 x 150). Posteriormente, se realizó un pre-procesamiento bioinformático de las lecturas generadas, tanto de la población OCF2 derivada de la cruza *eca2* x Col-0, como de la población derivada de la retrocruza, población BCF2 y del parental (fondo genético) de la mutante *eca2*. Se analizó la calidad de las lecturas mediante el software FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Las regiones de baja calidad y los adaptadores de secuenciación fueron filtradas y removidos usando el programa Trimmomatic (Bolger et al., 2014). Posteriormente, las secuencias se

alinearon al genoma de Arabidopsis (https://www.arabidopsis.org), TAIR versión 10 utilizando el programa Bowtie2 v2.3.5 (Langmead y Salzberg, 2012). Los archivos de alineación resultantes se convirtieron a archivos bam utilizando el paquete samtools v0.1.18 (Li et al., 2009) y se utilizaron como entrada para los subsiguientes análisis de búsqueda de SNP. Los archivos resultantes, fueron analizados con el programa bioinformático SHOREmap v3.0 instalado en el servidor kukulcan del CCG-UNAM; trabajando con las funciones pre-establecidas en el programa, *outcross* y *backcross* para encontrar la región de baja recombinación en el genoma de la población de mapeo, ubicando polimorfismos asociados a la mutación causante del fenotipo, presentes en el 100 % de las lecturas provenientes de la población con el fenotipo mutante; siendo esta, la región candidata (Schneeberger et al., 2009; Sun y Schneeberger, 2015). La **Figura 11**, muestra el flujo de trabajo con la herramienta SHOREmap que se siguió para el análisis.

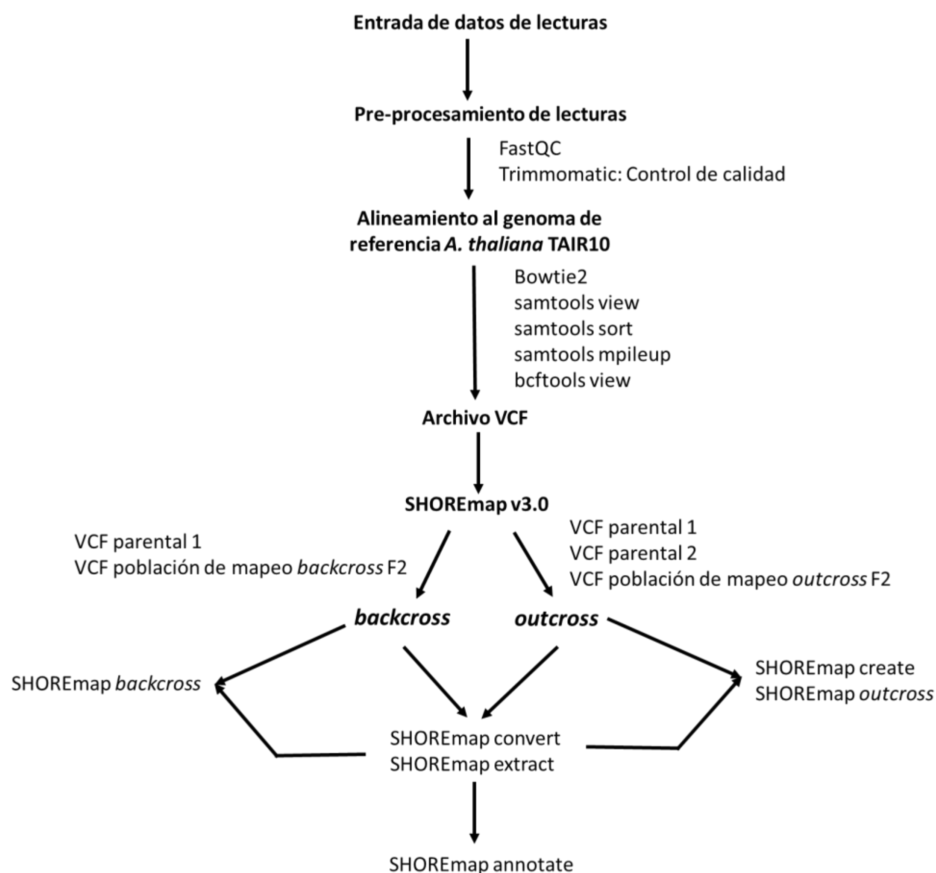

Figura 11. Flujo de trabajo para la búsqueda de SNP a partir de una población con fenotipo mutante obtenida por EMS, utilizando la herramienta SHOREmap opciones, *backcross* y *outcross*.

IV.3 Resultados.

IV.3.1 Generación y secuenciación de la población de mapeo.

Con la finalidad de ubicar principalmente a los SNP generados por el mutágeno EMS, en lugar de aquellos que son específicos de los ecotipos Col-0 (con quien se cruzó la mutante) y C24 (fondo genético de la mutante), se realizó la retrocruza de la mutante *eca2* con su parental tipo silvestre. Se seleccionó el tejido de 379 plántulas que presentaron el fenotipo silvestre y 127 el fenotipo de la mutante *eca2* (hojas azules), de un total de 516 plántulas crecidas bajo condiciones *in vitro* (**Figura 12**). De acuerdo al análisis genético, se presentó una segregación de 3:1 (**Tabla 2**), confirmando la naturaleza recesiva de la mutación en *eca2*, que anteriormente había sido determinada por Serrano y Guzmán en el 2004.

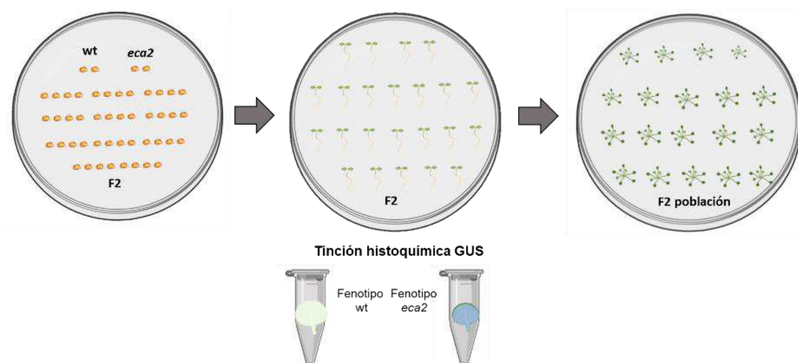

Figura 12. Diagrama de selección en condiciones *in vitro* de la población F2 segregante, derivada de la cruza entre *eca2* X wt, para obtener la población de mapeo. Se sembraron semillas de la mutante *eca2* y de la wt como control y semillas de plantas F2 segregantes. A los 10 días de edad se les hizo la tinción histoquímica de GUS. Las plántulas F2 que mostraron el fenotipo de *eca2*, se dejaron crecer hasta 20 días para su selección.

Tabla 2. Análisis genético de la mutante *eca2* en plántulas.

Cruza (hembra X macho)	Generación	No. de plantas	Patrón de expresión: wt	Patrón de expresión: *eca2*	χ^2
wt/wt X eca2/eca2 (wt X *eca2*)	F1	5	5	0	
wt/wt X eca2/eca2 (wt X *eca2*)	F2	516	379	127	0.6614; P < 0.05

χ^2 fue calculada de acuerdo con una proporción esperada 3:1 (wt x *eca2*).

IV.3.2 Identificación de genes candidatos causantes del fenotipo de *eca2*.

Para la búsqueda de genes candidatos causantes del fenotipo en la mutante *eca2*, se utilizaron dos estrategias. Una de ellas consistió en analizar las secuencias derivadas

de la cruza entre *eca2* y Col-0 (población OCF2); mientras que la otra se basó en generar la población de mapeo y la secuenciación del conjunto de plántulas segregantes F2, derivadas de la retrocruza entre *eca2* x planta tipo silvestre (población BCF2). El principio del método se muestra en la **Figura 13**.

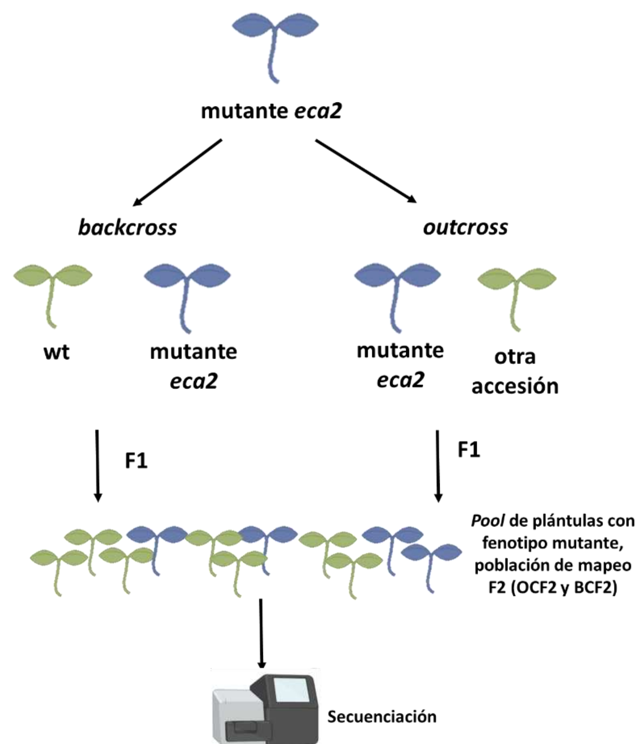

Figura 13. Esquema de las estrategias de mapeo e identificación de mutaciones en *eca2*. La mutante *eca2* en el fondo del ecotipo C24, puede cruzarse con la planta tipo silvestre (wt) para generar una población de mapeo derivada de una retrocruza o *backcross* (BCF2). O bien, cruzarse con una planta de diferente ecotipo para generar una población de mapeo tipo *outcross* (OCF2). Las plántulas segregantes que muestran el fenotipo mutante, se seleccionan para la extracción de ADN y posterior secuenciación de su genoma. Imagen adaptada de James et al. (2013).

Con respecto a la población de mapeo derivada de la retrocruza (BCF2), la secuenciación produjo un total de 43,513,442 de lecturas pareadas, para el parental tipo silvestre (100 nt), y 47,850,793 de lecturas pareadas (150 nt), para la población de mapeo segregante, con el fenotipo de la mutante *eca2*. El pre-procesamiento de las lecturas con los *softwares* FASTQC y Trimmomatic, resultó en la retención de la mayoría de estas. El total de lecturas, 39,162,097 y 44,979,745 del parental y de la población BCF2, respectivamente, se alinearon correctamente al genoma de referencia TAIR10. Se generaron los archivos con extensión .vcf tanto del parental, como de la población de

mapeo BCF2, sin embargo, al momento de implementar los algoritmos de SHOREmap función *backcross*, no fue posible identificar una región clara que estimara la frecuencia alélica dentro de la población de mapeo BCF2.

De manera paralela, se analizaron las secuencias derivadas de la población de mapeo OCF2. Se originaron 43,454,063 (100 nt) de lecturas de la población de mapeo OCF2, mientras que para el parental de tipo silvestre, se generaron 47,850,793 de lecturas pareadas (150 nt), y 41,283,096 de lecturas pareadas (150 nt) para las plantas del ecotipo Col-0 diferente al fondo genético de la mutante, las cuales fueron tomadas del trabajo desarrollado por Wang y colaboradores, (2021). Las lecturas se alinearon correctamente al genoma de referencia TAIR10. Se generaron los archivos con extensión .vcf de la población de mapeo OCF2, del parental tipo silvestre y de Col-0. Se utilizaron las herramientas SAMtools para la detección independiente de las diferencias entre la secuencia de referencia y las secuencias de la población con fenotipo mutante. Estos archivos se analizaron en mayor profundidad utilizando SHOREmap.

SHOREmap proporcionó la frecuencia alélica de los cambios inducidos por EMS en *loci* particulares en cada cromosoma (Chr). La FA se calcula en el programa como la proporción de los alelos de la mutante, dividido entre las lecturas en ese *locus* particular. En el conjunto de la población segregante, se espera que el cambio ocurra con la frecuencia más alta, por lo que todas las mutaciones inducidas por EMS deberían mostrar una FA alta. Por lo anterior, para los análisis subsecuentes se consideró una frecuencia alélica superior a 0.9. En la estrategia de *outcross* se introduce un gran número de polimorfismos naturales en el genoma de la población de las segregantes recombinantes, por lo que se toma como ventaja para la estimación de la frecuencia alélica que se usará para la predicción de genes candidatos de la mutación que causa el fenotipo. La visualización de la frecuencia alélica en cada cromosoma de Arabidopsis reveló una cercanía a 1 en el brazo superior del cromosoma 1, asociada a los cambios inducidos por EMS en la población que mostró el fenotipo mutante. La región mapeada que contiene a los genes candidatos de la mutación en el cromosoma 1, es atípicamente grande, comparada con los reportes en otras mutantes, sin embargo, modificando algunos parámetros entre los marcadores utilizados para el análisis, se logró reducir esta

región y limitar el número de genes identificados como candidatos con cambios inducidos por el EMS (**Figura 14**).

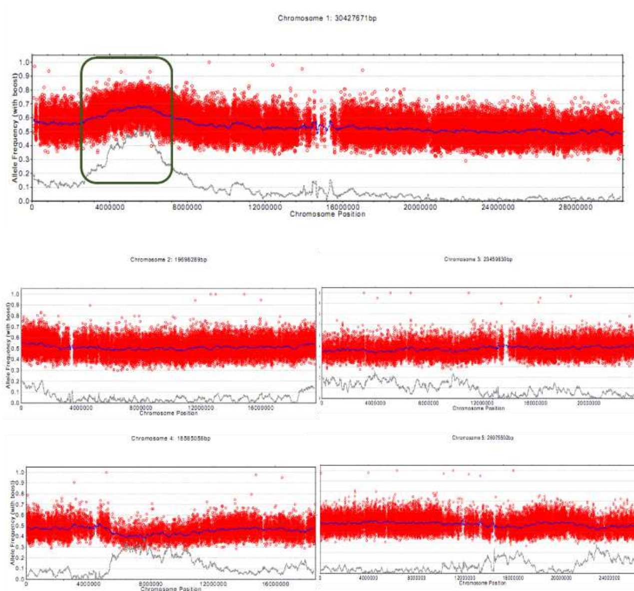

Figura 14. Visualización de la estimación de la frecuencia alélica en una población de mapeo clásica (*outcross*). Los puntos rojos indican la FA de acuerdo a lo estimado en los marcadores individuales (de los parentales y de la población de mapeo). La línea azul muestra la FA promedio dentro del intervalo de 400 kb. La línea negra se refiere a un parámetro (valor r) de la herramienta SHOREmap, que co-localiza la región que alberga la mutación que causa el fenotipo, con la más alta FA asociada a cambios inducidos por EMS. El rectángulo señala a esta región.

De este análisis se determinó que los genes candidatos con una mayor probabilidad de causar el fenotipo de la mutante *eca2* son AT1G16110 (***WAKL6,*** del inglés *wall associated kinase-like* 6), el cual codifica para receptores tipo **WAK**, que participan en la detección de la integridad de la pared celular, así como también en desencadenar diversas respuestas hacia patógenos mediante el sensado o percepción de PAMP o DAMP (Kohorn, 2015; Amsbury, 2020). Se ha reportado que plantas mutantes *wakl* no resultan afectadas en sus respuestas tempranas PTI (activación de cascadas de señalización de MAPKs y la producción de especies reactivas de oxígeno, ante la presencia de patógenos bacterianos; sin embargo, muestran afectación para activar respuestas tardías, como la deposición de callosa (Rosli et al., 2013; Kohorn et al., 2014; Rui y Dinneny, 2020). Asimismo, consideramos el gen AT1G14520 (*MIOX1*, del inglés *myo-inositol oxygenase* 1), que codifica para una *myo*-inositol oxigenasa (MIOX), toda vez que esta enzima, es clave en el proceso de síntesis del ácido UDP-D-glucurónico

(UDP-GlcA), principal precursor de los residuos de ácido galacturónico, xilosa, hemicelulosa y arabinosa, principales polímeros de la pared celular (Loewus, 2006; Klinghammer y Tenhaken, 2007). En Arabidopsis se han identificado 4 genes *MIOX* (MIOX1, MIOX2, MIOX4 y MIOX5). Interesantemente, los mutantes *miox2* exhiben un defecto en la biosíntesis de la pared celular (Kanter et al., 2005).

Finalmente, otro gen considerado es AT1G12630, que codifica para un miembro de la familia de factores de transcripción ERF/AP2, dado a que juegan un papel fundamental en la regulación de múltiples interacciones planta-patógeno, desencadenando cascadas de señalización para prevenir la infección (Heyman et al., 2018). El resumen de los genes identificados como candidatos para la mutación de *eca2*, se puede ver en la **Tabla 3**.

Tabla 3. SNP en los genes identificados como candidatos para la mutación en *eca2*.

Chr	Posición	Nucleótido en la referencia	Nucleótido en la mutante	FA	Región donde fue identificada la mutación	Gen	Tipo de mutación	Aminoácido en la referencia	Aminoácido en la mutante	
1	4,185,460	C	T	0.8	CDS	**AT1G12300**	Nonsyn	S	N	RFL2 is a pentatricopeptide repeat protein involved in mitochondrial RNA processing.
1	4,299,129	G	A	0.9	CDS	**AT1G12630**	Nonsyn	G	Q	A member of the DREB subfamily A-4 of ERF/AP2 transcription factor family.
1	4,806,612	C	T	0.86	CDS	**AT1G14030**	Nonsyn	D	N	LSMT-L, LYSINE METHYLTRANSFERASE (LSMT)-LIKE
1	4,968,508	G	A	0.71	CDS	**AT1G14520**	Nonsyn	T	I	Myo-inositol oxygenase 1(MIOX1)
1	5,137,957	G	A	0.88	3´UTR	**AT1G14890**		-	-	Pectin methylesterase inhibitor superfamily protein
1	5,519,695	G	A	0.76	CDS	**AT1G16110**	Nonsyn	R	Q	Wall associated kinase-like 6(WAKL6)
1	5,626,425	G	A	0.89	3´UTR	**AT1G16480**		-	-	Tetratricopeptide repeat (TPR)-like
1	5,662,360	G	A	0.8	CDS	**AT1G16540**	Nonsyn	G	R	ABA DEFICIENT 3 (ABA3), ACI2, ATLOS5, GIN5, LOS5, SIR3.
1	5,677,217	G	A	0.84	CDS	**AT1G16610**	Nonsyn	A	V	ARGININE/SERINE-RICH 45, ATSR45, RNPS1, SR45
1	5,690,540	G	A	0.83	CDS	**AT1G16650**	Nonsyn	R	Q	S-adenosyl-L-methionine-dependent methyltransferases
1	5,849,051	G	A	0.86	CDS	**AT1G17110**	Nonsyn	T	M	Ubiquitin-specific protease 15(UBP15)
1	7,988,883	C	T	0.85	CDS	**AT1G22600**	Nonsyn	G	D	Late embryogenesis abundant protein (LEA) family protein
1	7,679,578	G	A	0.84	CDS	**AT1G21870**	Nonsyn	G	R	Golgi Nucleotide Sugar Transporter 5, GONST5

Con la lista de genes resultante del análisis y seleccionados de acuerdo al fenotipo de *eca2*, una de las estrategias a seguir, primeramente, es la caracterización de líneas mutantes homocigotas por inserción (T-DNA) de cada uno de los genes, mediante el análisis de expresión del gen candidato, análisis de expresión del gen *ATL2*, permeabilidad de la cutícula, producción de especies reactivas de oxígeno y reto ante los patógenos *B. cinerea* y *P. syringae*. Una vez confirmado alguno de estos genes, se requiere la posterior complementación genética en la mutante *eca2*.

4. Conclusiones generales.

Con respecto a la interacción mutantes-*B. cinerea*:

- Las mutantes afectadas en el contenido de ceras *cer1-4* y *cer3-6*, presentaron un fenotipo susceptible a *B. cinerea,* a pesar de tener una cutícula permeable a solutos y producción de ROS. Demostrando así que estas características no son suficientes para inducir otras respuestas inmunes de la planta; contrario a la resistencia reportada para las mutantes de cutina *bdg, lacs2-3* y *eca2*, con cutícula permeable y producción de ROS.

- En las mutantes afectadas en el contenido de cutina *bdg*, *lacs2-3* y *eca2*, no se encontró un perfil de inducción de genes marcadores canónicos de defensa y de genes involucrados en las vías de señalización relacionadas con hormonas vegetales, tales como el SA, JA, ET o ABA, en respuesta a *B. cinerea*. Sugiriendo que el fenotipo de resistencia observado, puede deberse a la activación de vías de defensa distintas a las ya reportadas en mutantes similares.

- Se identificó la inducción de nuevos genes, como parte de los genes de defensa contra *B. cinerea*, en las mutantes de cutina *bdg*, *lacs2-3* y *eca2*, que no habían sido reportados en mutantes similares. Entre ellos se encuentran *AtPME17* y *AtPME41*, ambos codifican para pectin-metil-esterasas (PME); involucrados en la remodelación de la pared celular; genes del metabolismo de ROS como AT1G20620 (*CAT3*) que codifica para una catalasa; y AT5G64120 (*PRX71*) que codifica para una peroxidasa de clase III; y genes que codifican para factores de transcripción como AT1G43160 (*RAP2.6*) y At5g13330 (*ERF113*).

- En este trabajo se reportó por primera vez un perfil transcriptómico de mutantes afectadas en el contenido de ceras como *cer1-4* y *cer3-6*, tanto en presencia como en ausencia del hongo patógeno *B. cinerea*.

Con respecto a la identificación genética de la mutante *eca2*:

- El programa SHOREmap fue útil para analizar los datos de secuenciación del genoma de una población de mapeo F2 (OCF2), derivada de una cruza entre la mutante *eca2* y Col-0; y logró definir una región que alberga la mutación.

- La región se ubicó en el brazo superior del cromosoma 1 y se localizaron a los siguientes genes con mayor probabilidad de ser candidatos para causar el fenotipo de *eca2*: AT1G16110 (*WAKL6*) un gen que codifica para una tipo cinasa asociada a la pared celular; AT1G14520 (*MIOX1*) codifica para una *myo*-inositol oxigenasa; y AT1G12630 que codifica para un factor de transcripción de la familia ERF/AP2.

5. Perspectivas.

Con respecto a la interacción mutantes-*B. cinerea*:

- Dado a que se encontró la inducción de nuevos genes compartidos entre las mutantes afectadas en el contenido de cutina *bdg*, *lacs2-3* y *eca2*, durante la interacción con *B. cinerea*, entre ellos *AtPME17*, *AtPME41*, *CAT3* y *PRX71*; es indispensable caracterizar el fenotipo de líneas mutantes de cada uno de los genes, principalmente, evaluar variables como permeabilidad de la cutícula, composición química de la cutícula, producción de ROS y resistencia al hongo; con la finalidad de explorar el papel de estos genes en la inmunidad mediada por monómeros de cutina y si hay un vínculo con genes involucrados en la remodelación de la pared celular, así como con genes del metabolismo de ROS.

- Como continuación a este trabajo, resultaría interesante evaluar el papel del gen *PRX71*, en *bdg*, *lacs2-3* y *eca2,* en la producción de ROS observada de manera basal y en interacción con *B. cinerea* y su resistencia. Lo anterior, sumado a las evidencias que se tienen en otros trabajos, donde la mutante *qua2,* como punto a resaltar es que presenta un fenotipo alterado en la composición de la pared celular, principalmente en los componentes de pectina; además de la acumulación de ROS asociado con altos niveles de expresión del gen. Considerando lo anterior, se plantea generar dobles mutantes, que incluyan al gen *PRX71* y al de cada una de estas (*prx71-bdg*, *prx71-lacs2-3* y *prx71-eca2*); para caracterizar su fenotipo.

- Debido a que se comparte entre las mutantes afectadas en el contenido de cutina en interacción con *B. cinerea,* la inducción del gen *PRX71,* junto con *AtPME17* y *AtPME41*, cuyo fenotipo de permeabilidad de la cutícula y alto nivel de expresión de estos genes, es similar al de la mutante *qua2*, resulta interesante explorar los componentes de la pared celular, entre ellos la pectina. Se conoce recientemente, que mutantes con alterada composición de pectina, de celulosa o de lignina, han mostrado resistencia a *B. cinerea* y a otros hongos necrótrofos. Para ello, se propone la extracción y cuantificación de las fracciones que componen a la pared celular, a través de técnicas químicas y bioquímicas. Es importante también, compararlo con el perfil de las mutantes afectadas en el contenido de ceras *cer1-4* y *cer3-6*, que mostraron un fenotipo de susceptibilidad a *B. cinerea*.

- En vista que las mutantes afectadas en el contenido de cutina y en el contenido de ceras, mostraron un fenotipo diferencial hacia *B. cinerea*, resulta pertinente comprender el proceso de infección del hongo en sus etapas iniciales, en interacción con estas plantas. Para ello, se plantean dos estrategias, una de ellas es utilizar una cepa del hongo marcada con la proteína verde fluorescente (GFP) (Bc*gfp1*), y seguirla mediante microscopía confocal. La segunda estrategia consistiría en utilizar la cepa del hongo sin marcaje y seguirla mediante la técnica de microscopía electrónica de barrido (SEM). En ambas estrategias se proponen los tiempos 6 y 72 hpi.

- Por otra parte, se plantea explorar sobre el papel que juegan los microorganismos de la filósfera en las mutantes afectadas en el contenido de cutina *bdg*, *lacs2-3* y *eca2;* y de ceras *cer1-4* y *cer3-6*, en presencia de *B. cinerea*. Para lo cual se sugiere realizar experimentos de Metagenómica de cada una de ellas e identificar si existen microorganismos en común, en cada grupo y si la composición de la cutícula influye en el tipo de microorganismos encontrados y en la resistencia o susceptibilidad hacia el hongo.

Con respecto a la identificación genética de la mutante *eca2*:

- Corroborar e identificar el gen causante de la mutación de *eca2*, utilizando la estrategia de **complementación genética**. Para tal fin, se caracterizan genéticamente líneas mutantes por inserción por T-DNA, de cada uno de los genes con mayor probabilidad de causar el fenotipo de *eca2,* e identificar individuos homocigotos, para después caracterizarlos fenotípicamente, evaluando diferentes variables, como permeabilidad de la cutícula, ensayos de acumulación de ROS, y ensayos de infección con el hongo *B. cinerea* y con la bacteria *P. syringae*.

- Resultaría interesante explorar la estrategia de complementación química, toda vez que nuestra hipótesis, apuesta por los genes como causantes del fenotipo de *eca2*, a AT1G16110 (*WAKL6*) y a AT1G14520 (*MIOX1*), implicados en la síntesis de algunos componentes de la pared celular. En este sentido, el ensayo para el gen AT1G16110, se propone abordarlo mediante el uso de moléculas comerciales y constituyentes de la pared celular como oligogalacturónidos (OG). De la misma forma, para el gen AT1G14520 se propone el uso de ácido galacturónico. Se recomienda suplementar con estas moléculas el medio de crecimiento de la mutante *eca2*, en condiciones *in vitro*; y verificar el fenotipo que es de esperarse, una cutícula no permeable, similar al de la planta tipo silvestre.

CAPÍTULO V.
Colaboraciones.

De manera paralela al trabajo de investigación doctoral, se participó haciendo trabajo experimental para otros proyectos del grupo. De estas colaboraciones resultaron las siguientes publicaciones (archivos adjuntos). El trabajo de ambas, me permitieron conocer e implementar algunas de las técnicas que posteriormente utilicé en esta tesis doctoral.

1) Batista-Oliveira, J. S., Formey, D., Torres, M., Aragón, W., Romero-Contreras, Y. J., Maruri-López, I., Tromas, A., Schwan-Estrada, K., & Serrano, M. (2021). Gadolinium protects *Arabidopsis thaliana* against *Botrytis cinerea* through the activation of JA/ET-induced defense responses. *International journal of molecular sciences*, 22(9), 4938.

2) Ferreira-Saab, M., Formey, D., Torres, M., Aragón, W., Padilla, E. A., Tromas, A., Sohlenkamp, C., Schwan-Estrada, K., & Serrano, M. (2018). Compounds released by the biocontrol yeast *Hanseniaspora opuntiae* protect plants against *Corynespora cassiicola* and *Botrytis cinerea*. *Frontiers in microbiology*, 9, 1596.

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Gadolinium Protects *Arabidopsis thaliana* against *Botrytis cinerea* through the Activation of JA/ET-Induced Defense Responses

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Abstract: Plant food production is severely affected by fungi; to cope with this problem, farmers use synthetic fungicides. However, the need to reduce fungicide application has led to a search for alternatives, such as biostimulants. Rare-earth elements (REEs) are widely used as biostimulants, but their mode of action and their potential as an alternative to synthetic fungicides have not been fully studied. Here, the biostimulant effect of gadolinium (Gd) is explored using the plant-pathosystem *Arabidopsis thaliana–Botrytis cinerea.* We determine that Gd induces local, systemic, and long-lasting plant defense responses to *B. cinerea*, without affecting fungal development. The physiological changes induced by Gd have been related to its structural resemblance to calcium. However, our results show that the calcium-induced defense response is not sufficient to protect plants against *B. cinerea*, compared to Gd. Furthermore, a genome-wide transcriptomic analysis shows that Gd induces plant defenses and modifies early and late defense responses. However, the resistance to *B. cinerea* is dependent on JA/ET-induced responses. These data support the conclusion that Gd can be used as a biocontrol agent for *B. cinerea*. These results are a valuable tool to uncover the molecular mechanisms induced by REEs.

Keywords: *Arabidopsis thaliana*; biostimulant; *Botrytis cinerea*; defense responses; gadolinium; rare-earth elements

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1. Introduction

Two major factors determine the quantity and quality of plant-derived food: growth and development in the field and postharvest handling and storage conditions. Once a product is harvested, damage induced by microorganisms can cause 25% to 50% of the food to be lost [1]. Fungi of the genera *Alternaria, Aspergillus, Botrytis, Fusarium, Geotrichum, Gloeosporium, Penicillium, Mucor*, and *Rhizopus* are responsible for most of these losses [2,3]. In particular, the ubiquitous necrotrophic fungus *Botrytis cinerea* (Pers) has been shown to infect more than 200 plant species and to be responsible for "botrytis bunch rot" or "grey mold" symptoms. Due to these characteristics, *B. cinerea* has been classified as the second most important phytopathogen in existence [3]. To reduce the damage caused by *B. cinerea* and other fungi, farmers use different chemicals, including fungicides and biostimulants. The latter have been proposed as a new eco-friendly alternative to synthetic fungicides and are defined as naturally occurring molecules, elicitors, or microorganisms that enhance plant development, abiotic and biotic stress resistance, and/or crop quality traits [4,5].

Biostimulants that activate plant defense responses include polypeptides; glycoproteins; lipids; proteins; glycolipids; oligosaccharides; and microbe-, herbivore-, and damage-

associated molecular patterns [4–6]. Defense responses primed by biostimulants include the activation of the plant innate immunity and the late defense responses. As part of the immune response, the accumulation of reactive oxygen species (ROS), calcium (Ca^{2+}) influx, protein phosphorylation, mitogen-activated protein kinase (MAPK) signaling, and transcriptional induction of the early defense response genes take place [7–9]. After the initial response, the secondary defense responses are activated, including the production of histological barriers such as callose or lignin and the induction of salicylic acid—(SA), jasmonic acid—(JA), and ethylene—(ET) dependent signaling pathways [10]. These pathways finally lead to the induction of systemic acquired resistance (SAR) in non-infected distal organs of the plant [6,11,12]. The combined effect of these defense responses can efficiently stop disease induced by non-adapted pathogens, including fungi.

Rare-earth elements (REEs) are trace metals from the lanthanide group. While REEs are not known to be nutritionally essential to plants, they have been used as biostimulants in agriculture, particularly in China, to improve plant growth and development [13–16]. Depending on their concentration, REEs can have positive and negative effects on plant growth, development, and production (see reviews [17,18]). For instance, the application of a low concentration of neodymium (1, 3 or 5 mg/L) improved the germination rates of *Cassia obtusifolia* seeds, while higher concentrations inhibited them [19]. On the other hand, the application of 6 mg/L of neodymium increased the germination rates of *Astragalus membranaceu* seeds up to 42% [18], suggesting that the beneficial effects of REEs depend not only on the concentration but also on the plant species. Nevertheless, the beneficial effects of REEs have been related to their structural chemical resemblance to the secondary messenger, calcium [20]. Many REEs can displace the divalent Ca cation due to their trivalent charges and thus higher charge density, modifying an increased number of Ca-mediated biological processes [21]. In particular, calcium channels have been reported to be specifically blocked by the so-called "super calcium molecule" gadolinium, modifying multiple plant responses to abiotic and biotic stimuli [22,23]. Nevertheless, the effect of REEs on plant biology still remains mostly unknown [17]. For this reason, it is still not clear if the biological effects of REEs are only due to their structural chemical resemblance to Ca or to as-yet unknown mechanisms.

Nowadays, food production is based on the application of fertilizers—for instance, 18.8×10^{10} Kg of phosphate-based fertilizers are applied every year worldwide [24]. REEs are found as minor components of the raw material of phosphate-based fertilizers (monazites) [25,26]. For this reason, REEs are constantly applied to plants as biostimulants and/or as part of fertilizers, although basic information about them, such as their molecular mechanisms and the transcriptional changes induced by their application, are mostly unknown. On the other hand, their possible use as an alternative to synthetic fungicides has not been fully explored either. Only a handful of reports have studied the effect of REEs during plant–microbe interactions, some with contradictory outcomes. Some of these studies have indicated that the application of REEs induces defense responses against brown blast disease in rubber trees and fusarium wilt in tomato [27,28], while another report showed their inhibition of plant defense mechanisms [29]. Nevertheless, in order to become an alternative to synthetic fungicides, it is first necessary to properly characterize the molecular changes induced by REEs and then optimize their application and activity in the field.

In this report, we showed that the REE gadolinium (Gd) is a biostimulant that improves the growth and development of *A. thaliana* roots. Additionally, we determined that Gd protects plants against the necrotrophic fungus *B. cinerea*, without affecting the development of the fungus itself. Gd has a dose-dependent and long-lasting effect, triggering local and systemic defense responses against *B. cinerea*. To our knowledge, this is the first report of change in the plant transcriptome induced by Gd during the interaction with *B. cinerea* using genome-wide analysis (RNA-seq). This information will be a valuable tool to uncover the molecular mechanisms induced by REEs and will help in future efforts to improve and fully exploit their use in agriculture.

2. Results

2.1. Gadolinium Improves Root Development of A. thaliana

Our previous results indicated that the application of 0.2 g/L of gadolinium (III) nitrate hexahydrate ($Gd(NO_3)_3 \cdot 6H_2O$) has a positive effect on the development of *Glycine max* compared to plants treated with 0.2 g/L of calcium nitrate ($Ca(NO_3)_2$), henceforth identified as Gd and Ca [30]. Based on this observation and in order to better understand the molecular mechanisms induced by the exogenous application of gadolinium, we analyzed its effect on the model plant *A. thaliana*. *A. thaliana* seeds were germinated in the presence of distilled sterile water (as mock), 0.2 g/L Ca, or 0.2 g/L Gd. Ca was also used as a control because of its structural resemblance to Gd. Ten days after germination, a significant increase in root length was observed in Gd-treated plants compared to mock- and Ca-treated samples (Figure 1A). Afterwards, the same seedlings were transplanted to soil and the number of leaves was counted 30 days later. Once more, we observed a slight increase in the number of leaves in Gd-induced samples compared to the mock- and Ca-treated samples. However, these differences were not statistically significant (Figure S1). Additionally, no changes in germination rate or fresh or dry weight were observed between the mock-, Ca-, and Gd-treated samples (Figure S1). Taken together, these results suggest that Ca did not affect *A. thaliana* growth and that exogenous Gd application improved the root growth of *A. thaliana*.

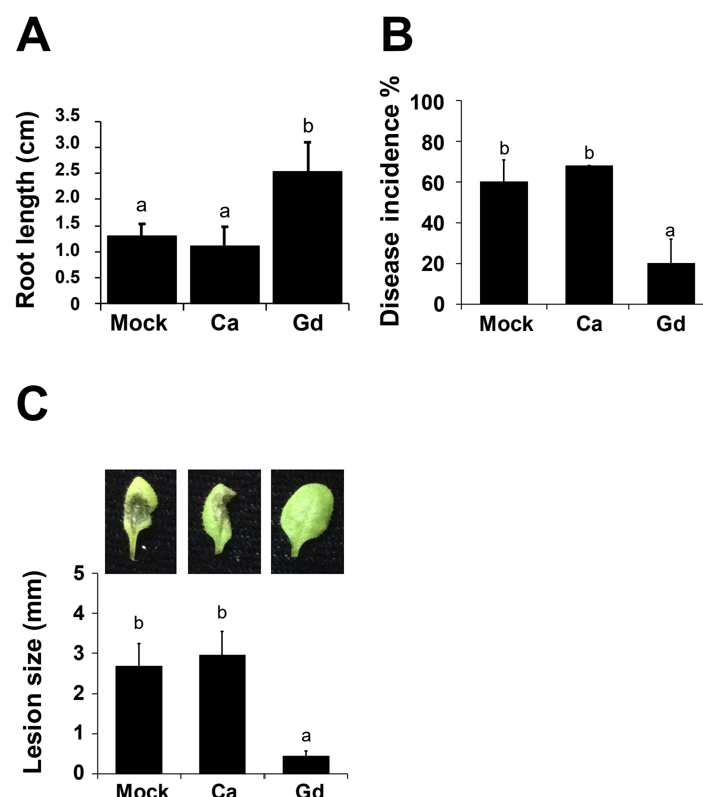

Figure 1. Gd improved the root development of *A. thaliana* and induced protection against *B. cinerea*. *A. thaliana* seeds were incubated for 1 h with distilled sterile water (mock), 0.2 g/L Ca, or Gd and germinated under in vitro conditions. Ten days post germination, the primary root length was measured (**A**). Four-weeks-old *A. thaliana* plants were sprayed until saturation with distilled sterile water (mock), 0.2 g/L Ca, or Gd (24 hpt). After this, 3 µL droplets containing a *B. cinerea* spore suspension (5×10^4 spores mL^{-1}) were applied and the 72 hpi disease incidence (**B**) and lesion size (**C**) were evaluated. A representative picture is included above each histogram as a visual illustration. Bars represent mean values (±SD) of three independent experiments ($n = 30$ for each experiment). Different letters above each bar represent statistically significant differences according to the Scott–Knott test ($p < 0.05$).

2.2. *Gadolinium Protects Arabidopsis thaliana Plants against the Necrotrophic Pathogen B. cinerea*

Our next step was to determine if Gd has an effect in the well-characterized plant-pathosystem *A. thaliana–B. cinerea*. Four-week-old *A. thaliana* plants were pre-treated for 24 h (hpt) by spraying with 0.2 g/L Gd or Ca and distilled sterile water (as mock) and afterwards infected with *B. cinerea* spores. First, we measured the percentage of plants showing disease symptoms 72 h post infection (hpi), expressed as disease incidence (Figure 1B). No statistical differences were observed between the mock- and Ca-treated plants, suggesting that Ca did not affect the *A. thaliana–B. cinerea* interaction. However, we observed a 50% reduction in incidence in Gd-treated plants compared to Ca-treated control samples (Figure 1B). Additionally, when we characterized the disease severity by measuring the lesion size at 72 hpi, plants treated with Gd showed a strong reduction in the infection (almost to 80%) compared to the control Ca-treated samples (Figure 1C). Taken together, these results indicate that the exogenous application of Gd causes a strong reduction in disease incidence and infection severity from the necrotrophic pathogen *B. cinerea*. Additionally, since we determined that Ca did not affect the *A. thaliana–B. cinerea* interaction compared to H_2O-treated plants (Figure 1B,C) and in order to eliminate the possible phenotypes induced by the structural resemblance between Ca and Gd, in the rest of the experiments we decided to use only Ca as a mock control.

2.3. *Gd Does Not Affect the Development of B. cinerea*

REEs have been shown to inhibit the development of several microorganisms [31,32]. To determine if the protective effect observed in plants (Figure 1C) was triggered either by the direct effect of Gd localized on the leaf surface or by the induction of the plant defense responses, we characterized the development of the pathogen in the presence of Gd under in vitro and in planta conditions (Figure 2). Spore suspensions, at a final concentration of 5×10^4 spores per ml, were grown on a Petri dish containing PDA media supplemented with water (mock); 0.2 g/L Ca; or Gd at 0.2, 0.4, 0.8, and 1.6 g/L and incubated under optimal growth conditions for 10 days. We determined that Gd did not inhibit the growth of *B. cinerea* mycelium at any of the treatments (Figure 2A). Additionally, to determine if Gd has an effect on the development of *B. cinerea*, the number of spores was quantified. Once more, no statistical differences were observed at either of the two concentrations (0.2 and 1.6 g/L Gd) nor at 0.2 g/L Ca, compared to the mock-treated samples (Figure 2B). To further characterize these observations, the fungal growth in planta was followed by trypan blue staining. At 24 hpi, no differences in the hyphal development between mock-, Ca-, and Gd-treated leaves were observed. However, at 48 hpi the hyphal growth of *B. cinerea* on Gd-induced leaves was inhibited (Figure 2C). This suggests that the gemination of the spores was not modified, and while the initial compatible interaction between *B. cinerea* and *A. thaliana* might not be affected by Gd application, the progression of the infection is inhibited. Taken together, these results suggest that Gd induces a protective effect, as previously observed in planta (Figure 1), which could be mediated by the modifications of the plant defense responses rather than a direct effect on fungal growth and development.

2.4. *Gd-Induced Protection against B. cinerea Is Dose-Dependent and Long-Lasting*

To determine the optimal concentration of Gd-induced resistance against *B. cinerea*, a dose-dependent experiment was performed. We inoculated 4-week-old *A. thaliana* plants pre-treated for 24 hpt with 0.05, 0.1, 0.2, 0.4, and 0.8 g/L Gd, as well as 0.8 g/L Ca as a control, with *B. cinerea* spores, and measured the lesion size at 72 hpi (Figure 3A). No protection against the pathogen was observed on plants treated with the lowest concentration of Gd (0.05 g/L, Figure 3A). However, samples pre-treated with 0.1 to 0.8 g/L of Gd showed a strong reduction in lesion size of approximately 70%, compared to Ca-treated control plants (Figure 3A). It is noteworthy that plants pre-treated with 1.6 g/L of Gd showed spontaneous lesions and, for this reason, were not included in the analysis. These results show that a reduction in lesion size through the exogenous application of Gd is

dose-dependent, with a strong inhibition of disease already occurring at a concentration of 0.1 g/L of Gd.

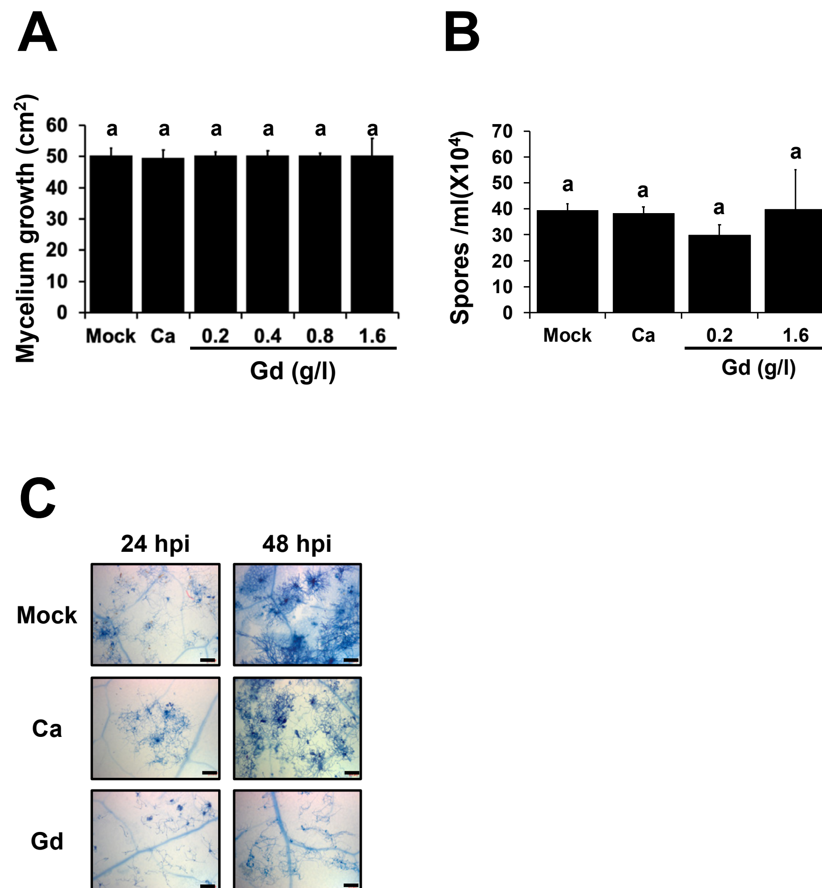

Figure 2. Gd does not affect the growth and development of *B. cinerea*. (**A**) A total of 5 µL of spore suspension of *B. cinerea* (5×10^4 spores mL^{-1}) was placed on the center of the Petri dish containing PDA supplemented with water (mock), 0.2 g/L Ca, and the indicated concentrations of Gd and incubated at 22 °C. Growth inhibition was evaluated by measuring the diameter of the mycelium on the dish 3 days post inoculation. (**B**) Spores produced by *B. cinerea* 10 days after growth on a Petri dish containing PDA supplemented with mock, 0.2 g/L Ca, and the indicated concentrations of Gd were isolated and quantified as previously described [33]. (**C**) *B. cinerea* development over *A. thaliana*-infected leaves was determined by trypan blue staining at 24 and 48 hpi. Representative images were selected as a visual illustration from two independent experiments. Bars represent mean values (±SD) of three independent experiments. Scale bar = 200 µm. Different letters above each bar represent statistically significant differences according to the Scott–Knott test ($p < 0.05$).

To evaluate for how long Gd can protect *A. thaliana* plants against *B. cinerea*, different pre-treatment times were analyzed by measuring the lesion size at 72 hpi. Interestingly, the Ca-treated control plants showed a significant reduction in lesion size at 48, 72, and 96 hpt of approximately 30% compared to 24 and 120 hpt, suggesting that Ca has an effect on the plant–pathogen interaction at these time points (Figure 3B). Nevertheless, at all the time points analyzed, Gd-treated plants always showed a significant reduction in lesion size compared to their corresponding control Ca-treated samples, fluctuating between 60% (24 and 120 hpt) and 40% (48, 72, and 96 hpt) (Figure 3B). Thus, these results indicate that Gd exerts a protective effect against *B. cinerea* over several days.

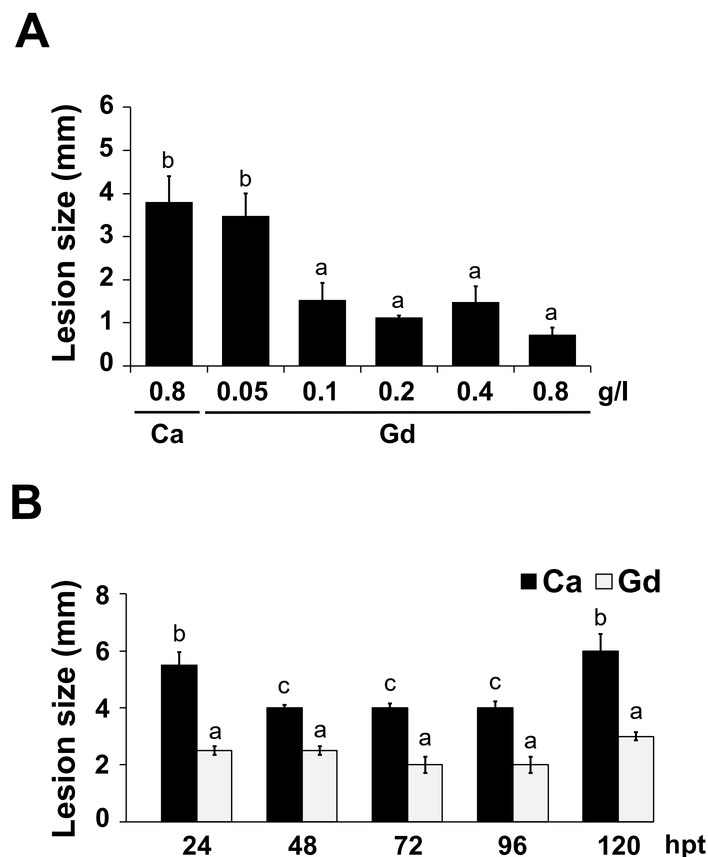

Figure 3. Gd-induced protection against *B. cinerea* is dose-dependent and long-lasting. (**A**) Four-week-old *A. thaliana* plants were pre-treated for 24 h (24 hpt) with the indicated Gd concentration or 0.8 g/L of Ca. After this, 3 μL droplets containing a *B. cinerea* spore suspension (5×10^4 spores mL^{-1}) were applied and infection symptoms were evaluated at 72 hpi by measuring lesion size. (**B**) Four-week-old *A. thaliana* plants were sprayed until saturation with 0.2 g/L of Ca or Gd for 24, 48, 72, 96, or 120 (hpt); after these times, 3 μL droplets containing *B. cinerea* spore suspension (5×10^4 spores mL^{-1}) were applied and infection symptoms were evaluated at 72 hpi by measuring lesion size. Bars represent mean values (±SD) of three independent experiments (n = 30 for each experiment). Different letters above each bar represent statistically significant differences according to the Scott–Knott test ($p < 0.05$).

2.5. Gd Triggers a Systemic Defense Response to B. cinerea

To further characterize the Gd-induced plant defenses against *B. cinerea*, we analyzed whether a systemic protection was activated (Figure 4). Firstly, half of the rosette leaves from 4-week-old *A. thaliana* plants were pre-treated with 0.2 g/L of Gd or Ca (local) and the other half with H_2O (systemic). Then, at 24 hpt we inoculated all the leaves with *B. cinerea* spores and measured the lesion size at 72 hpi. Plants treated with Gd showed a strong reduction in the lesion sizes for both tissues (local and systemic) compared to their respective Ca-treated control leaves (Figure 4A). In another tray, 4-week-old *A. thaliana* plants were watered to saturation with a solution of 0.2 g/L of Gd or Ca and untreated leaves were inoculated for 24 hpt with *B. cinerea* and the lesion size measured at 72 hpi. Gd-watered plants showed an approximately 50% reduction in lesion size compared to the Ca-watered control plants (Figure 4B). These results indicate that Gd induces a systemic defense response against *B. cinerea* in *A. thaliana* plants.

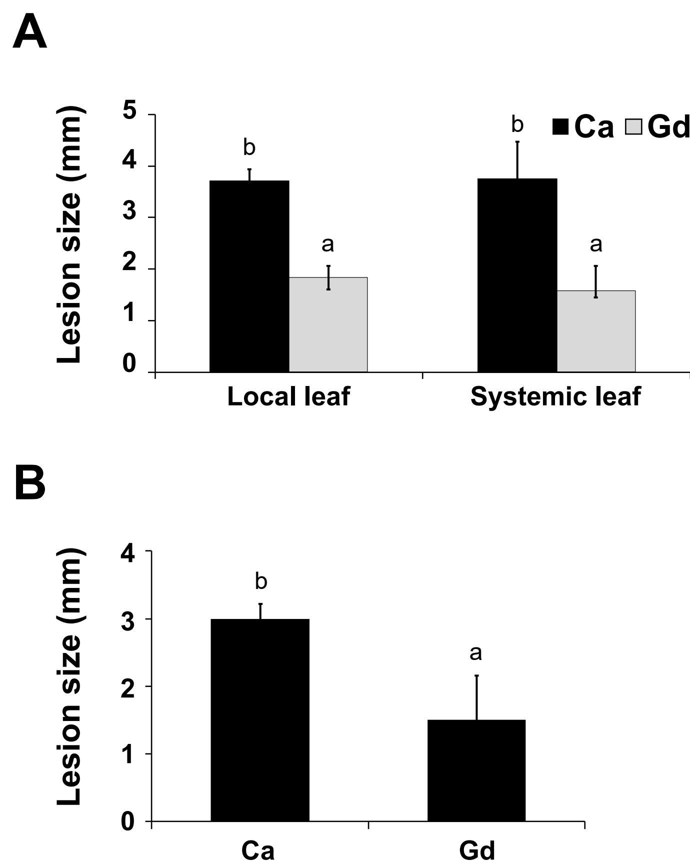

Figure 4. Systemic effect induced by Gd against *B. cinerea* in *A. thaliana* plants. Four-week-old *A. thaliana* plants were pre-treated as follows: (**A**) half of the rosette leaves were pre-treated with 0.2 g/L Gd or Ca (local) and the other half with H_2O (systemic); (**B**) plants were watered until saturation with 0.2 g/L of Gd or Ca. At 24 hpt, 3 µL droplets containing *B. cinerea* spore suspension (5×10^4 spores mL^{-1}) were applied. Infection symptoms were evaluated at 72 hpi by measuring lesion size. Bars represent mean values (±SD) of three independent experiments (n = 30 for each experiment). Different letters above each bar represent statistically significant differences according to the Scott–Knott test ($p < 0.05$).

2.6. Gd Does Not Modify the Cuticle Permeability but Triggers a ROS Burst

We have described that cuticle-related mutants, as part of a defensive syndrome, showing an increase in leaf permeability and a rapid induction of the plant innate immunity, including the accumulation of reactive oxygen species (ROS) and resistance against *B. cinerea* [34,35]. In order to determine if the Gd-triggered defense responses against *B. cinerea* are mediated by a similar syndrome, we quantified the leaf permeability using three different methods: measuring the efflux of chlorophyll, toluidine blue staining, and calcofluor staining, as previously described [33]. Four-week-old *A. thaliana* plants were treated for 24 h with 0.2 g/L of Gd or Ca. Leaf permeability was not modified by exogenous Gd application, since no differences were observed between Ca- and Gd-treated samples in any of the methods used (Figure S2). Then, using a similar treatment, we quantified the presence of ROS at 0, 3, 6, 12, and 24 hpt (Figure 5) through histological analysis and densitometric quantification with the probe 5-(and-6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCF-DA), which detects a broad spectrum of ROS. Histological analysis showed that, at 3 hpt, a strong accumulation of ROS induced by Gd (Figure 5A) was already detected and further maintained without significant changes up to 24 hpt, while with Ca-treated plants ROS was not induced at any of the time points (Figure 5A). These observations were confirmed by densitometric quantification (Figure 5B). Taken together, these

results suggest that the ROS burst detected after Gd application was induced directly by Gd treatment rather than by a modification of the cuticle permeability.

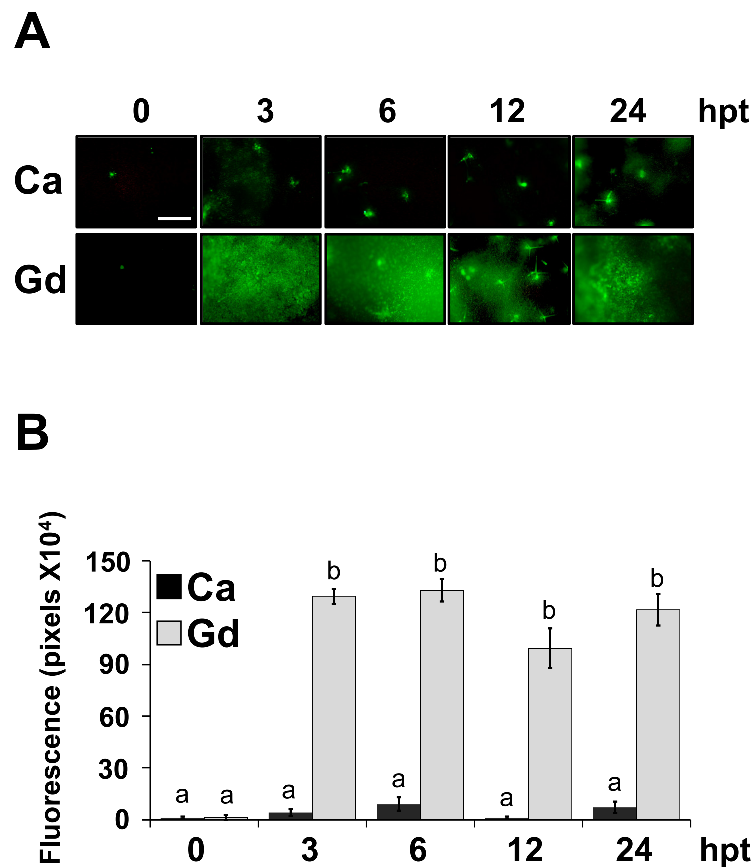

Figure 5. Gd-induced accumulation of ROS. (**A**) Leaves from 4-week-old *A. thaliana* plants treated with 0.2 g/L of Ca or Gd were stained using DCF-DA to detect basal ROS levels at the indicated time point (**B**) Densitometric quantification of ROS production at indicated time points after Ca or Gd treatment. Bars represent the mean values (±SD) of three independent experiments ($n = 30$). Different letters above each bar represent statistically significant differences according to the Scott–Knott test ($p < 0.05$). Scale bar = 200 µm.

2.7. Gadolinium Up-Regulates the Responses to Biotic Stimulus and Represses the Responses to Abiotic Stimulus

To identify the genetic elements that participate in Gd-induced responses, we studied the transcriptional changes in the Gd-treated plants using a genome-wide RNA sequencing analysis (RNA-seq). Total RNA was isolated from 4-week-old *A. thaliana* plants at two different time points—first at 24 hpt with 0.2 g/L of Gd or Ca and then from plants at 24 hpi inoculated with *B. cinerea* (Figure 6). Gd-pretreated plants showed 1189 and 200 genes with significantly higher and lower expressions, respectively, compared to the Ca-induced ones (Figure 6A; Supplementary Data 1). Gd-treated plants that were infected with *B. cinerea* showed 673 and 433 genes with higher or lower expressions, respectively, compared to Ca-treated plants (Figure 6B; Supplementary Data 2). These results suggest that the application of exogenous Gd modifies the transcriptome to improve the growth and defense responses of *A. thaliana*.

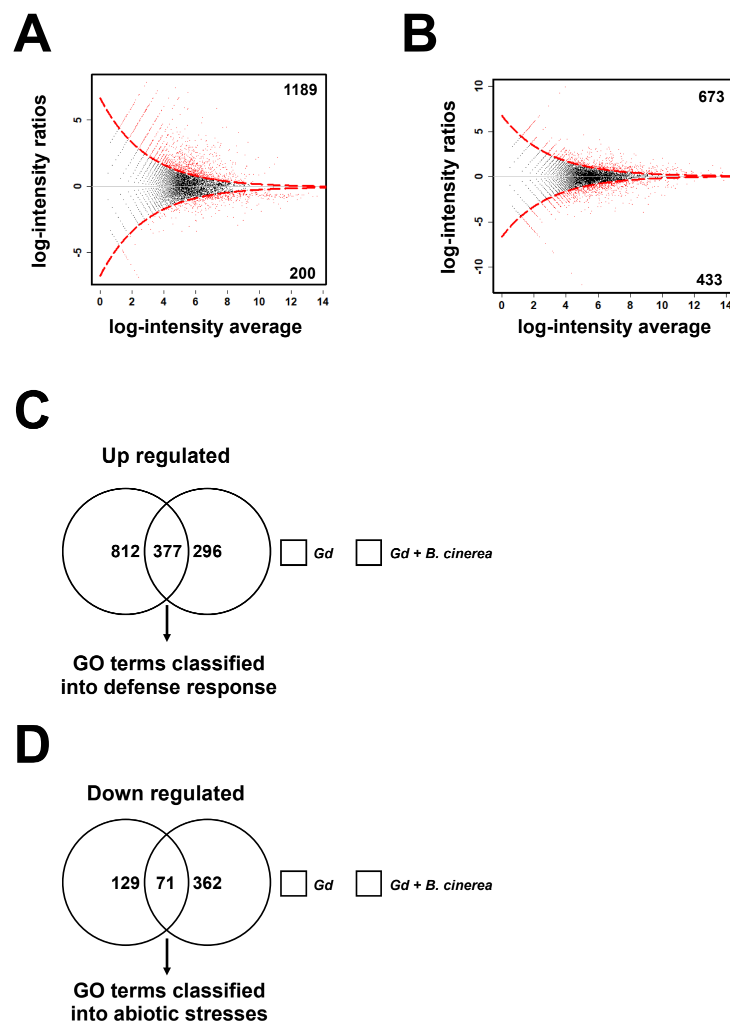

Figure 6. RNAseq analysis of Gd-induced *A. thaliana* plants. Five 4-week-old *A. thaliana* plants were sprayed until saturation with 0.2 g/L of Ca or Gd (24 hpt) and then infected with *B. cinerea* (24 hpi). Total RNA for each condition, from three independent experiments, was pooled and sequenced (RNA-seq). (**A**) MA plot of Gd- versus Ca-treated samples at 24 hpt. (**B**) MA plot of Gd- versus Ca-treated samples infected with *B. cinerea* at 24 hpi. The red points represent DEGs (p-value < 0.05), while black dots indicate genes with a similar expression. The dotted red line shows the limit between similarly and differentially expressed genes. The black horizontal line at zero provides a visual check for symmetry. Venn diagrams representing overlapping or non-overlapping gene sets of up-regulated DEGs (**C**) and down-regulated genes (**D**), respectively, were identified in *A. thaliana* plants induced with 0.2 g/L of Ca or Gd and infected with *B. cinerea*, as indicated.

From the DEGs detected in the Gd application conditions compared to Ca (Table S1), we wanted to identify those that had higher and lower expressions after the infection by *B. cinerea* took place. A total of 377 induced genes and 71 repressed genes follow this pattern (Figure 6C,D). Interestingly, the GO analysis of these DEGs revealed that almost all the genes with a higher expression were classified into the defense response mechanisms, including responses to fungi and innate immune responses (Table S1). In contrast, the repressed genes were classified mostly in the category of responses to abiotic stresses (Table S1). These results suggest that different sets of mechanisms are induced or repressed by Gd application.

2.8. Plant Defense Response Genes Are Induced by Gadolinium Treatment and during the Interaction with B. cinerea

The transcriptional activation of the defense responses induced by Gd was analyzed using the bioinformatics tool MapMan [36]. From this analysis, an induction of the responses to biotic stress was observed in Gd-pretreated plants (24 hpt) (Figure S3A) and Gd-induced plants later infected with *B. cinerea* (24 hpi) (Figure S3B). A list of candidate genes that have been characterized as improving the resistance against *B. cinerea* was recently described [10]. We analyzed the accumulation of these candidate transcripts in Gd-induced plants (24 hpt) and the plants after the interaction with the pathogen (24 hpi) in comparation to Ca-treated samples (Figure 7A). Eight out of 13 genes were induced at 24 hpt, while 10 were up-regulated at 24 hpi, suggesting that Gd transcriptionally induces defense responses against *B. cinerea*. To further confirm and validate the genome-wide RNA sequencing analysis (Supplementary Data 1 and 2), we further characterized by qRT-PCR the accumulation of transcripts of representative *A. thaliana* genes previously described to be involved in the plant–microbe interactions from the ROS—(*ZAT12*) [37], SA—(*ICS1* and *PR1*) [38,39], JA—(*PDF1.2*) [40], and ET-signaling pathways (*ACS6* and *PR4*) [41,42]. This was conducted in 4-week-old *A. thaliana* plants pretreated with H_20 (mock), Ca, or Gd (24 hpt) (Figure 7B). Remarkably, we determined that the mock- and Ca-treated samples showed similar expression patterns for all the genes, confirming the validity of the Ca control. On the other hand, we observed that at 24 hpt, all the analyzed genes were induced in Gd-treated plants compared to the Ca-induced ones. It is worth mentioning that for all the genes analyzed, the expression signatures were similar to those detected by RNAseq (highlighted in Supplementary Data 1). Additionally, based on the structural similarities between Gd and Ca, one might expect that Ca-induced genes will be also up-regulated by Gd. In agreement with this, several Ca-responsive genes were part of the Gd-induced ones, such as the calcium-binding EF-hand family protein (AT3G01830) and the vacuolar calcium-binding protein-related (AT1G62480) (Supplementary Data 1), validating our analysis. Taken together, these results suggest that application of Gd transcriptionally activates the selected ROS-, SA-, JA-, and ET-induced defense response genes that were previously identified as part of the plant defense mechanisms against *B. cinerea* [33,43].

2.9. Gd-Induced Defense Response Against B. cinerea Is Dependent of JA and ET

As part of the defense response against *B. cinerea*, the plant transcriptome is modified, including the activation of ROS-, SA-, ET-, ABA-, JA-, and ET-induced signaling pathways [45]. In this work, we show that Gd transcriptionally induces ROS-, SA-, JA-, and ET-induced defense response genes (Figure 7). In order to determine the effect of each response pathway on Gd-triggered defenses, we analyzed mutants impaired in ROS, SA, JA, and ET accumulation. Measuring the disease incidence and lesion size, we determined that plants were still protected against *B. cinerea* after Gd application in the mutants of *NADPH oxidase D* (*AtrbohD*) and *F* (*AtrbohF*) involved in ROS production (Figure 8A,B). On the other hand, even the SA-deficient mutant *eds5* showed a slight increase in disease incidence and lesion size after Gd application, although these changes were not statistically significant compared to those in Col-0 wt-treated plants (Figure 8A,B). However, for the ET- and JA-related mutants *ethylene-insensitive* 3 (*ein3*), *jasmonate-amido synthetase* (*jar1*), and *lipoxygenase* 2 (*lox2*), we observed a similar disease incidence and lesion sizes in Gd-induced plants compared to the Ca-treated samples, suggesting that the Gd-induced defense response is mediated by JA and ET (Figure 8A,B). To confirm this observation, we selected the genes from our transcriptomic analysis that had been previously described to be involved in ET- and JA-related responses that have also been characterized to be involved in resistance to *B. cinerea* [46], and we analyzed their differential accumulation status (Figure 8C,D). For ET-related genes, 60% were induced at 24 hpt with Gd, while 30% were up-regulated after the interaction with the pathogen took place (24 hpi, Figure 8C). On the other hand, for JA-related genes 78% were induced after the treatment with Gd (24hpt) and 48% kept this expression pattern after the interaction with *B. cinerea* (24 hpi)

(Figure 8D). Taken together, these results indicated that the Gd-triggered defense response against *B. cinerea* is mostly dependent on the JA/ET-induced responses.

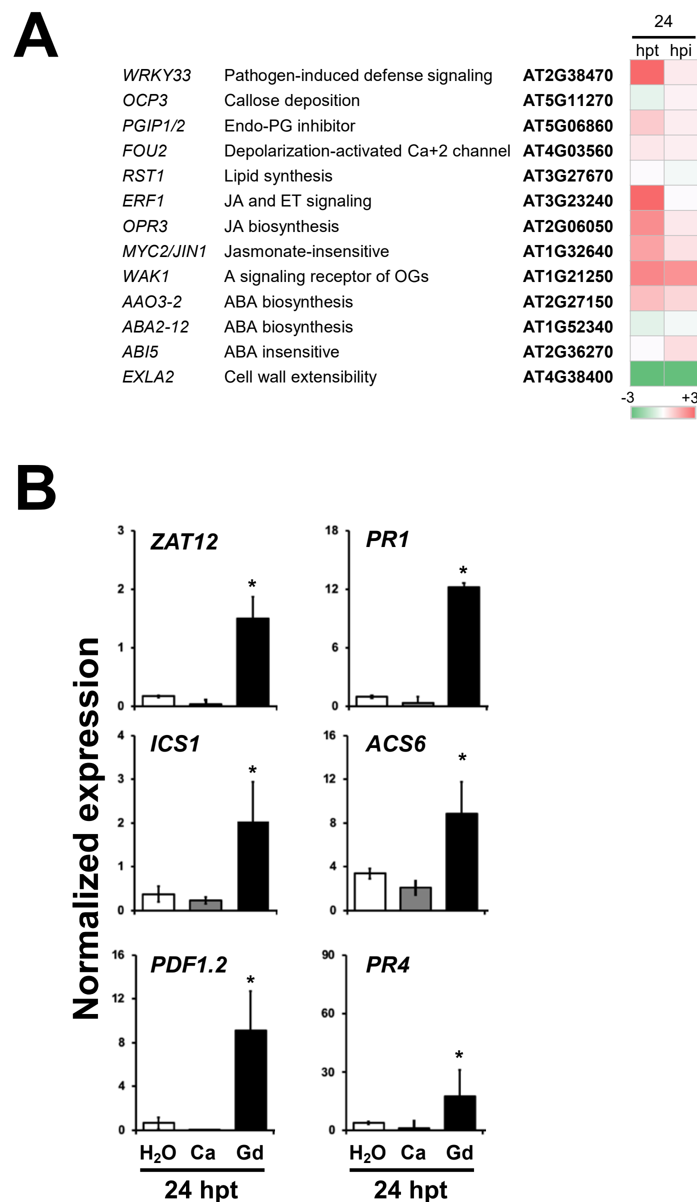

Figure 7. Gd transcriptionally activate the plant defense response genes. (**A**) Heatmaps of the expression log2-fold changes of genes previously described to be involved in the resistance against *B. cinerea* [10], compared to the corresponding controls, from RNAseq data at 24 hpt and hpi. (**B**) Four-week-old *A. thaliana* plants pretreated with H_2O (mock), 0.2 g/L Ca, or Gd (24 hpt). Quantitative real-time PCR (qRT-PCR) expression analysis of *ZAT12*, *ICS1*, *PR1*, *ACS6*, *PDF1.2*, and *PR4* was determined and normalized with respect to the mean of two reference genes *AT4G26410* and *AT1G72150*, as previously described [43,44]. A representative experiment with three technical replicates is shown (±SE). Three independent experiments were carried out with similar results. Asterisk above each bar represents statistically significant differences to the mock-treated samples according to the Student's *T*-test ($p < 0.05$).

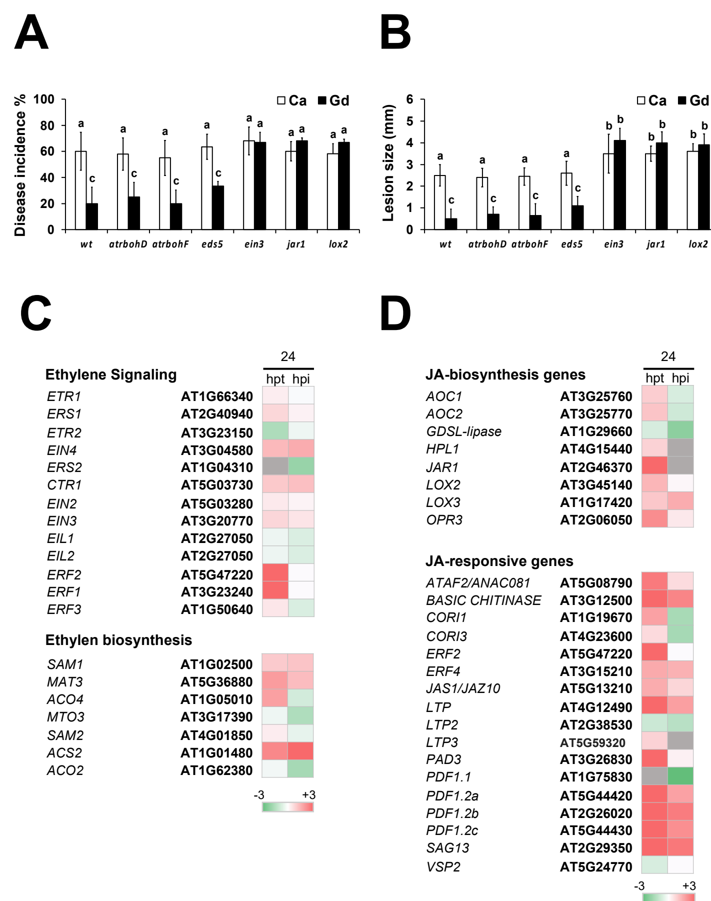

Figure 8. Role of ROS, SA, and JA in Gd-induced resistance to *B. cinerea* in *A. thaliana*. The *AtrbohD, AtrbohF, eds5, ein3, jar1*, and *lox2* mutants were evaluated. (**A**) Four-week-old *A. thaliana* plants were sprayed until saturation with 0.2 g/L of Ca or Gd. After 24 hpt, 3 μL droplets containing a *B. cinerea* spore suspension (5×10^4 spores mL^{-1}) were applied and disease incidence was determined by measuring the percentage of plants showing disease symptoms at 72 hpi. (**B**) The disease severity was determined by measuring the lesion size of all the infected leaves. Heatmaps of the expression of log2-fold changes in ET- (**C**) and JA-related genes (**D**), compared to the corresponding controls, from RNAseq data at 24 hpt and hpi. Bars represent mean values (±SD) of three independent experiments (n = 50). Different letters above each bar represent statistically significant differences according to the Scott–Knott test (p < 0.05).

3. Discussion

3.1. A Transcriptome Analysis Provides New Insights into the Molecular Mechanisms Involved in the Gd Response in A. thaliana

During the last 30 years, REEs have been widely used as biostimulants in the farmlands of China and other countries [47,48]. Previous physiological analyses indicated that the exogenous application of REEs modifies calcium-induced responses, such as the structure and function of the cytoplasmic membranes, photosynthesis, the modulation of hormone metabolism, and the increased efficiency of water use [48]. Nevertheless, their modes of action(s), in particular at the transcriptional level and during biotic interactions, are still poorly understood [49]. In this report, we show that *A. thaliana* treated with gadolinium (Gd) protected the plants against *B. cinerea* (Figure 1). In order to uncover the molecular mechanisms behind this effect, we performed a genome-wide transcriptional analysis (Supplementary Data 1). The GO analysis revealed that almost all the induced genes were classified into the defense response mechanisms, while the repressed ones were classified mostly in response to abiotic stresses (Figure 6). Interestingly, several processes which had been previously associated with changes in root growth were identified among

the repressed DEGs, including responses to abscisic acid (ABA) and water deprivation. High concentrations of ABA have been shown to inhibit root growth [50], while water deficiency stimulates it [51]. These reports support the view that the information generated in this work can be used as a starting point to unravel the molecular mechanisms behind REE-induced responses, potentially leading to the optimization of their application as biostimulants.

3.2. Gd Is a Novel Biocontrol against the Broad Host-Range Necrotrophic Fungus B. cinerea

One of the limiting factors in food production is disease caused by fungi [52], with *B. cinerea* being one of the major ones responsible for these losses [2]. Most of the treatments to control the infections inflicted by this pathogen are based on the application of synthetic fungicides. However, an increase in worldwide regulatory policies and the claim to reduce their application due to the possible harmful side effects have led to searches for new eco-friendly alternatives, such as biostimulants. Despite the generalized use of REEs as biostimulants in agriculture, only a few reports have studied its impacts on plant–microbe interactions [27–29], and to our knowledge none have described the inhibition or modification of the interaction with *B. cinerea*. Here, we show that Gd can protect *A. thaliana* plants against *B. cinerea* by inducing defense responses. We also show that even at high Gd concentrations, the growth and development of *B. cinerea* is not affected by Gd or Ca under in vitro or in planta conditions (Figure 2). This is somewhat unexpected, since REEs have been described as inhibiting the development of other microorganisms, and high concentrations of $CaCl_2$ can decrease the spore germination and mycelial growth of *B. cinerea* [53]. These results suggest that the protective effect induced by Gd is mediated by the modification of the plant defense mechanisms rather than a direct inhibitory effect on the pathogen. Additionally, we determined that the Gd-induced protection is dose-dependent and lasts for 5 days (Figure 3). All these results indicate that Gd has the potential to be used as a biocontrol against this agronomically important pathogen.

3.3. Gd Induces Protection against B. cinerea by Activating Early and Late Defense Responses, in Particular the JA- and ET-Induced Signaling Pathway

Our RNA-seq analysis shows that early and late defense responses are transcriptionally induced after the application of Gd (Table S1). As part of the early defense mechanisms, plants can trigger innate immunity, which includes the accumulation of reactive oxygen species (ROS), calcium (Ca^{2+}) influx, protein phosphorylation, MAPK-dependent signaling cascades, and the transcriptional induction of defense response genes [7,8]. In this work, we observed a fast accumulation of intracellular ROS after Gd application (3 hpt), which was further maintained for 24 h (Figure 5). This is consistent with the fact that we have described a direct link between ROS accumulation and the resistance to *B. cinerea* in previous studies [33,34,54]. This could be sufficient to explain the reduction in infection observed in Gd-treated plants. However, it is necessary to mention that other REEs have been evidenced to modify ROS accumulation [55]. Additionally, we determined that mutants of the enzymes *AtrbohD* and *AtrbohF*, involved in intracellular ROS production, can still be protected after Gd treatment (Figure 8), suggesting that either only intracellular ROS is produced or that other genes are probably implicated in Gd-induced ROS production. It would be interesting to test this hypothesis using mutants deficient in extracellular ROS production. Based on this information, we cannot discard the idea that the ROS burst might be triggered specifically by Gd or as a general response to incubation with REEs, and can only assume that this is part of the defense mechanism against *B. cinerea*.

The activation of innate immunity is followed by secondary mechanisms, including the induction of SA-, JA-, and ET-dependent signaling pathways, finally leading to the activation of systemic acquired resistance (SAR) in non-infected distal parts of the plant [6,11,12]. We revealed that SA-, JA-, and ET-induced genes were transcriptionally activated after Gd treatment and during the interaction with *B. cinerea* (Figure 7 and Supplementary Data 1 and 2). However, we observed that the mutant impaired in SA accumulation (*eds5*) shows a slight, but not statistically significant, reduction in Gd-induced protection. The protection

induced by this REE was completely lost in *ein3*, *jar1,* and *lox2* mutants, which are impaired in JA- and ET-induced responses (Figure 8). SA-triggered defense responses have been reported to be involved against necrotrophic pathogens in *A. thaliana* [56,57] and tobacco [58]. However, other studies have reported that *A. thaliana* defense responses against *B. cinerea* depend mostly on JA and ET [59–61]. Our results indicate that the protective effect of Gd against this pathogen depends on ET- and JA-dependent signaling pathways.

3.4. Ca might Elicit the Defense Responses but Not as Strongly as Gd

To cope with environmental stresses, plant responses are intimately coordinated by the complex signaling networks of the so called "trio signaling" messengers: ROS, electrical signals, and calcium [62]. In particular, Ca plays an important role in plant–pathogen interactions, since once an attack is perceived its intracellular concentration is rapidly increased, activating defense responses in a local and systemic fashion [63]. As mentioned, the physiological changes induced by REEs have been related to their structural chemical resemblance to calcium [20]. In agreement, Gd has been described to elicit a similar signaling pathway to this secondary messenger [64]. In this report, in order to reduce the expected phenotypes induced by the structural and functional resemblance of Gd and Ca, we used calcium nitrate as a control. Ca has been described to inhibit the spore germination and mycelial growth of *B. cinerea* [53]. Nevertheless, we did not detect a reduction in infection due to *B. cinerea* on *A. thaliana* plants treated with Ca (Figure 1B,C) either locally or systemically at this concentration (Figure 4). However, interestingly, during the time-course experiment, we observed a significant reduction in lesion size after 48, 72, and 96 hpt in Ca-induced samples, compared to at 24 hpt in Ca-treated plants (Figure 3B). This reduction, however, was never as strong as that measured in Gd-treated plants at any of the time points analyzed (Figure 3B). This result suggests that both Ca and Gd might elicit defense responses, though at different times, against the necrotrophic pathogen *B. cinerea*. A detailed analysis of the Ca-induced defense mechanisms after 48, 72, and 96 hpt and of all the DEGs identified in this work might help us to uncover and understand the potential differences between Ca- and Gd-induced defense pathways.

Finally, even if our initial goal was to characterize the effect of Gd on *B. cinerea*, it is worth mentioning that our RNA-seq analysis suggests that this REE might extend its effect over other biotic and abiotic stresses. Among the biological processes induced by Gd treatment, several defense responses were identified, including responses to other organisms, such as bacteria and response to salicylic acid (SA). For instance, the SA-induced signaling pathway is a central part of the plant defense responses to multiple pathogens, including infections by viruses, bacteria, and fungi [65]. On the other hand, our GO analysis revealed that repressed genes were classified mostly in response to abiotic stimuli, including responses to abscisic acid (ABA) and water deprivation. REEs have been described to increase plant resistance to different abiotic stresses [17,66]. With this in mind, our results seem to point towards a possible use of Gd in the context of sustainable agricultural production to enhance plant tolerance to numerous biotic and abiotic stresses. Nevertheless, to continue using Gd as a plant stimulator it will be necessary to take into account that long-term use might increase its potential as a source of pollution in the soil, as previously described for other REEs [67].

4. Materials and Methods

4.1. Plant Material and Growth Conditions

Arabidopsis thaliana (L.), Heynh ecotype Columbia-0 (Col-0), and *lox2* mutant were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK). The *A. thaliana* mutants used in this work (all in the Col-0 background) were the following: *AtrbohD*, *AtrbohF* [68], *eds5* [69] *jar1* [70], and *ein3* [71]. Plants grown under in vitro conditions were generated from surface-sterilized seeds germinated in square Petri dishes containing Murashige and Skoog (MS) media supplemented with 0.8% agar and 1% sucrose. Plates were kept at 4 °C for two days and incubated vertically at 21 °C for 10 days in a growth

chamber with a 16 h light/8 h dark photoperiod. For ex vitro experiments, *A. thaliana* seeds were grown on a pasteurized (applying heat until the mix reaches 82 °C for 30 min) soil mix of humus/perlite (3:1), kept at 4 °C for two days, and then transferred to the growth chamber. Plants were grown for 4 weeks in a 12 h light/12 h dark cycle with a 60–70% relative humidity, a light intensity of 200 µmol/m^2/s, a day temperature of 20–22 °C, and a night temperature of 16–18 °C. For in vitro experiments, surface-sterilized *A. thaliana* seeds were incubated for 1 h in distilled sterile water (mock), 0.2 g/L (0.0012 M) calcium nitrate ($Ca(NO_3)_2$), or 0.2 g/L (0.00044 M) gadolinium (III) nitrate hexahydrate ($Gd(NO_3)_3 \cdot 6H_2O$). Seeds were germinated as described above and the germination rate, primary root length, and fresh and dry weight were measured 10 days post germination.

4.2. Gadolinium Treatment and B. cinerea Plant Inoculation

Four-week-old *A. thaliana* plants were pre-treated by spraying to the point of run off with 0.2 g/L of gadolinium (III) nitrate hexahydrate, 0.2 g/L of calcium nitrate, or H_2O for 24, 48, 72, 96, or 120 h post-treatment, hpt. After this time, 3 µL droplets of *B. cinerea* spore suspension (5×10^4 spores mL^{-1}) were applied. The procedures of *B. cinerea* infection, including disease incidence and measurement of lesion size, were determined 72 h post-infection (hpi), as previously described [33]. For the dose–response assay, plants were pre-treated for 24 h (24 hpt) with the indicated concentration of gadolinium (III) nitrate hexahydrate or calcium nitrate and then infected with *B. cinerea* and evaluated at 72 hpi.

4.3. Systemic Defense Response Analysis

For the systemic assay, two methodologies were used: (i) half of the rosette leaves of 4-week-old *A. thaliana* plants were pre-treated by spraying with 0.2 g/L of gadolinium(III) nitrate hexahydrate or calcium nitrate (local) and the other half with H_2O (systemic) and (ii) 4-week-old *A. thaliana* plants were pre-treated (watering the soil until saturation) with 0.2 g/L of gadolinium(III) nitrate hexahydrate or calcium nitrate for 24 h (24 hpt). For both conditions, leaves were infected with *B. cinerea* and evaluated at 72 hpi, as described above.

4.4. In Planta B. cinerea Growth Analysis

Fungal hyphae were stained and plant cell death assayed as previously described [72]. Once stained, leaves were imbibed in 20% glycerol for 1 h and observed using a microscope with bright-field settings. Representative images were selected as a visual illustration.

4.5. In Vitro Inhibitory Assay of B. cinerea Growth

The *B. cinerea* strain BMM (originally isolated from grape wine) was provided by Brigitte Mauch-Mani (University of Neuchatel, Switzerland). *B. cinerea* in vitro growth and preparation of spore suspension were performed as previously described [33]. For the inhibition assay, 5 µL of a spore suspension of *B. cinerea* (5×10^4 spores mL^{-1}) was placed at the center of a Petri dish containing potato dextrose agar media (PDA) supplemented with 0.2 g/L of calcium nitrate or 0.2, 0.4, 0.8, or 1.6 g/L of gadolinium(III)nitrate hexahydrate, as indicated in the figure legends. The plates were incubated at 22 °C for 3 days and then the diameter of the mycelial growth was determined using Image J version 1.51 (U. S. National Institutes of Health, Bethesda, MD, USA), as previously described [73]. Ten days post incubation, *B. cinerea* spores were collected from the plates and re-suspended in 5 mL of H_2O and quantified using a Neubauer cell counting chamber.

4.6. Cuticle Permeability Analysis

We recently described that cuticle-related mutants show an increase in leaf permeability and a rapid induction of plant innate immunity, including the accumulation of reactive oxygen species (ROS) [34,35]. To determine the cuticle permeability, three methods were used: (*i*) calcofluor white staining, (*ii*) toluidine blue staining, and (*iii*) chlorophyll extraction and quantification [33,34,54]. To stain with calcofluor white, leaves were bleached in absolute ethanol overnight, equilibrated in 0.2 M $NaPO_4$ (pH 9) for 1 h, and incu-

bated for 1 min in 0.5% calcofluor white in 0.2 M $NaPO_4$ (pH 9). Leaves were rinsed in $NaPO_4$ buffer to remove excess calcofluor white and were viewed under a UV light. Toluidine blue staining was carried out by placing 6 μL droplets on the leaf surface of 0.025% toluidine blue solution dissolved in $\frac{1}{4}$ PDB. After incubation for 2 h, the leaves were washed gently with distilled water to remove excess solution from them. Representative images were selected as a visual illustration. For chlorophyll extraction and quantification, leaves were weighed and immersed in 30 mL of 80% ethanol. Chlorophyll suspension was sampled in the dark at room temperature with gentle agitation at 2, 5, 10, 20, 30, 40, 50, 60, and 120 min after immersion. The chlorophyll content was determined by measuring the absorbance at 664 and 647 nm and the micromolar concentration of total chlorophyll per gram of fresh weight of tissue was calculated using the following equation: $(7.93 \times (A_{664}\ nm) + 19.53 \times (A_{647}\ nm))\ g^{-1}$ fresh weight.

4.7. Detection of ROS

ROS were detected using the fluorescent probe 5-(and-6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCF-DA), as previously described [33]. Representative images were selected as a visual illustration.

4.8. RNA Extraction and Genome-Wide Transcriptomic Analysis

For the three independent experiments, leaves from 5 *A. thaliana* plants were harvested, pooled and immediately frozen in liquid nitrogen and kept at −80 °C. Two conditions were analyzed. The first was at 24 hpt with 0.2 g/L of gadolinium(III)nitrate hexahydrate or calcium nitrate and the second was from plants pre-treated by spraying the entire leaf with gadolinium(III)nitrate hexahydrate (24 hpt) or calcium nitrate and infected for 24 hpi with *B. cinerea* by spraying the spore suspension all over the leaf. Total RNA was extracted as described in the manufacturer's protocols using the Spectrum^{TM} Plant total RNA Kit (Sigma Aldrich, San Luis, MO, USA). The integrity of the extracted RNA was measured by agarose gel electrophoresis (1.2%). The NanoDrop 2000/2000c (Thermo Fisher Scientific, Waltham, MA, USA) was used to calculate concentrations and purity. Samples used for RNA-seq were also analyzed using an Agilent 2100 Bioanalyzer (Agilent Genomics, Santa Clara, CA, USA). RNA-seq libraries were prepared following the manufacturer's instructions from isolated total RNA using the Illumina TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). The libraries were sequenced using the manufacturer's protocol of the Illumina GAIIx platform for 72 paired-end cycles. Sequences are publicly available through the Gene Expression Omnibus database under the accession number GSE123522. Contamination and adapter removal were carried out using in-house Perl scripts. Fastq sequences were filtered based on quality (FASTQ Quality Filter v0.0.6, Q 33, http://hannonlab.cshl.edu/fastx_toolkit/index.html) and mapped to the *A. thaliana* transcriptome (TAIR10) using Bowtie2 [74]. Gene expression was determined using RSEM v1.3 [75] and compared between the RNA-seq libraries using DEGseq v3.6 [76] and the FPKM data from RSEM. Only transcripts with a Log2 fold change of <-1 or >1 with a p-value < 0.05 were considered. DEGs identified by genome-wide transcriptomic analysis were analyzed and classified into gene ontology classes (GO) using the PANTHER Gene List Analysis tools and default parameters. The identification of commonly regulated DEGs was performed using the software FiRe ver. 2.2, as previously described [77]. The MapMan software was used to visualize the amplitudes of the changes in the expression of individual genes in diagrams of cellular processes, as previously described [36].

4.9. Quantitative Real Time RT-PCR

The pooled total RNA (1.0 μg) used for RNAseq analysis was retro-transcribed into cDNA according to the manufacturer's indications using the SCRIPT cDNA Synthesis Kit (Jena Bioscience, Jena, Germany). qRT-PCR was performed in 96-well plates with the Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA) using the SYBR Green Maxima SYBR Green/ROX qPCR

Master Mix (2X) (ThermoFisher Scientific, Waltham, MA, USA). Three independent experiments were analyzed, each with three technical replicates. The qRT-PCR conditions were as follows: an initial 95 °C denaturation step for 5 min, followed by denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C for 45 cycles. Gene expression values were normalized using the mean expression of two genes: AT4G26410 (*RHIP1*) and AT1G72150 (*PATL1*), which were previously described as stable reference genes [44,78]. Normalized gene expression was determined using the comparative $2^{-\Delta\Delta CT}$ method, as previously described [79]. Primers for gene expression analysis have been previously described: for *PR1*, *PDF1.2* and *PR4* [43]; for *ZAT12* [37], *ICS1* [80] and *ACS6* [81].

4.10. Statistical Analysis

All results are reported as mean values (±SD) and were analyzed by an analysis of variance and compared by the Scott–Knott test ($p < 0.05$) using the software GraphPad Prism version 8.1.0 (2019, GraphPad Software, San Diego, CA, USA). All the data analyzed were obtained from three independent experiments.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22094938/s1, Supplementary Table S1: GO enrichment analysis of differentially expressed genes commonly induced or repressed after Gd application (24 hpt) and during the interaction with *B. cinerea* (24 hpi), Supplementary Figure S1: Gd does not affect germination rates, fresh nor dry weight, Supplementary Figure S2: Gd does not modify plant cuticle permeability, Supplementary Figure S3: MapMan pathway analysis of differentially expressed genes of (A) 24 hpt with 0.2 gL^{-1} Gd and (B) afterwards infected with with *B. cinereal* (24 hpi) compared to Ca-treated samples, Supplementary Data 1: List of significantly differentially expressed genes 24 hpt, Supplementary Data 2: List of significantly differentially expressed genes 24 hpi.

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Compounds Released by the Biocontrol Yeast *Hanseniaspora opuntiae* Protect Plants Against *Corynespora cassiicola* and *Botrytis cinerea*

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Plant diseases induced by fungi are among the most important limiting factors during pre- and post-harvest food production. For decades, synthetic chemical fungicides have been used to control these diseases, however, increase on worldwide regulatory policies and the demand to reduce their application, have led to searching for new ecofriendly alternatives such as the biostimulants. The commercial application of yeasts as biocontrol agents, has shown low efficacy compared to synthetic fungicides, mostly due to the limited knowledge of the molecular mechanisms of yeast-induced responses. To date, only two genome-wide transcriptomic analyses have characterized the mode of action of biocontrols using the plant model *Arabidopsis thaliana*, missing, in our point of view, all its molecular and genomic potential. Here we describe that compounds released by the biocontrol yeast *Hanseniaspora opuntiae* (HoFs) can protect *Glycine max* and *Arabidopsis thaliana* plants against the broad host-range necrotrophic fungi *Corynespora cassiicola* and *Botrytis cinerea*. We show that HoFs have a long-lasting, dose-dependent local, and systemic effect against *Botrytis cinerea*. Additionally, we performed a genome-wide transcriptomic analysis to identify genes differentially expressed after application of HoFs in *Arabidopsis thaliana*. Our work provides novel and valuable information that can help researchers to improve HoFs efficacy in order for it to become an ecofriendly alternative to synthetic fungicides.

Keywords: Biocontrol agent, elicitors, *Hanseniaspora opuntiae*, *Corynespora cassiicola*, *Botrytis cinerea*, *Glycine max*, *Arabidopsis thaliana*, plant defense responses

INTRODUCTION

Of all food produced for human consumption, every year 1.3 billion tons are lost or wasted (http://www.fao.org). Only during post-harvest, 25 to 50% of the production can be lost due to plant diseases induced by microorganisms and by suboptimal handling and storage conditions (Nunes, 2012). Fungal species are responsible for most of these losses, including the genera *Alternaria, Aspergillus, Botrytis, Fusarium, Geotrichum, Gloeosporium, Penicillium, Mucor*, and

Rhizopus (Barkai-Golan, 2001; Dean et al., 2012). The importance of fungi-related disease can be exemplified by mentioning that if producers could avoid the damages associated to fungi in the five most important crops, 600 million people could be fed each year (Fisher et al., 2012). For decades, fungicides have been used to control fungi-induced diseases. However, an increase in worldwide regulatory policies and the demand to reduce their application, due to potential harmful side effects to the environment and to humans, have led to searching for new ecofriendly alternatives. One of these alternatives is biostimulants, which are defined as a naturally-occurring chemicals or microorganisms that enhance plant development, abiotic, and biotic stress tolerance and/or crop quality traits (Du Jardin, 2015).

Biostimulants that protect the plant against pathogens can be classified as elicitors and biocontrol agents (BCAs). Microorganisms such as bacteria and yeast, have been used as BCAs to control herbivores and several plant pathogens. For instance, bacteria from the genera *Bacillus*, *Pseudomonas,* and *Pantoea* have been used to control mold-produced fungi, mainly by the production of antibiotics (Nunes et al., 2002; Cirvilleri et al., 2005; Ren et al., 2013). However, even if some of them are already used in the field, several concerns arrise, in particular the possible development of resistance in the pathogens. Nowadays, one alternative is to use yeast as BCAs, since they are antagonistic microorganisms that can grow under adverse environmental conditions without special nutrients requirements and do not produce compounds harmful to human health (Liu et al., 2013). The basis of the antagonistic properties of yeast against pathogens has been previously described and includes: competition for nutrients, pH changes on the plant surface, production of ethanol and biosynthesis of killer toxins called mycocins (Hatoum et al., 2012). Nevertheless, despite all these beneficial traits, the commercial application of yeast in the field as BCAs has shown an inconsistent efficacy compared to synthetic fungicides, mostly due to the lack of knowledge of the molecular mechanisms behind yeast-induced plant defense responses (Massart et al., 2015).

On the other hand, elicitors are chemical molecules that activate the plant defense responses, and include microbe- and damage-associated molecular patterns (MAMPs and DAMPs), polypeptides, glycoproteins, lipids, proteins, glycolipids, and oligosaccharides (Katagiri and Tsuda, 2010; Maffei et al., 2012; Hael-Conrad et al., 2015; Yin et al., 2016). Once the elicitors are perceived by the plant, the first line of defense, called plant innate immunity is activated. During this initial defense mechanism, the production of reactive oxygen species (ROS), calcium influx, MAPK-dependent signaling cascades, localized cell death and transcriptional induction of the early defense response genes are activated (Katagiri and Tsuda, 2010; Tsuda and Somssich, 2015). After the induction of innate immunity at the local infected tissue, secondary defense responses are triggered, including salicylic the acid- (SA), jasmonic acid- (JA), and ethylene- (ET) dependent signaling pathways, that lead to the activation of systemic acquired resistance (SAR) at non-infected distal parts of the plant (Boller and Felix, 2009; Robert-Seilaniantz et al., 2011). The combined effect of the local and systemic defense responses, can block efficiently the disease inflicted by non-adapted pathogens (Craig et al., 2009). Due to these characteristics, elicitors have the potential to be used in agriculture as alternative to fungicides. However, to do so, it is necessary to better characterize the molecular changes induced by elicitors in order to optimize its application and activity in the field (Wiesel et al., 2014).

Molecular characterization of the plant-microbe interactions has been greatly benefitted from the technical advances in areas including metabolomics, proteomics, genomics and bioinformatics, in particular using *Arabidopsis thaliana* as a model. For example, this has led to novel conceptual advances in the understanding of the molecular basis of plant-pathogen interactions (Mishra et al., 2017). Importantly, these advances also saw the dawn of a series of potential applications that could impact crop protection (Bhadauria, 2016). During the last decade, several genome-wide transcriptomic analyses have been used to characterize the mode of action of BCAs (Massart et al., 2015). However, strangely, many of these analyses have been performed under *in-vitro* conditions and only two of them were characterized using the interaction *Arabidopsis thaliana*-BCAs as pathosystem (Feng et al., 2012; Morán-Diez et al., 2012).

HoFs

In this report, we show that compounds released by the biocontrol yeast *Hanseniaspora opuntiae,* henceforth identified as *H. opuntiae*-Filtrates (HoFs), have the potential to protect against the broad host-range necrotrophic fungi *Corynespora cassiicola* and *Botrytis cinerea.* In order to better understand the molecular basis of HoFs-induced resistance, we characterized its activity in the well-described pathosystem *Arabidopsis thaliana-Botrytis cinerea*. We determined that HoFs can protect *Arabidopsis thaliana* against the necrotrophic fungus *Botrytis cinerea*. HoFs can induce the defense response in a dose-dependent manner. Additionally, performing a genome-wide transcriptomic analysis (RNA-seq), we identified that the genes differentially expressed upon application of HoFs, differ from those induced by other previously-described BCAs. This valuable information might help to reveal the molecular mechanisms behind HoFs-induced defense and can help researchers to improve their efficacy and to become an ecofriendly alternative to pesticides.

MATERIALS AND METHODS

Purification of HoFs

Hanseniaspora opuntiae CCMA 0760, was provided by the laboratory of Physiology and Genetics of the Federal University of Lavras, Brazil. *Hanseniaspora opuntiae* was grown in YNB (Yeast Nitrogen Base) media for 10 days in a 12 h light/12 h dark cycle at 24°C. At the end of the growth period, the culture media was centrifuged at 10,000 rpm for 20 min and the supernatant was filtered using 0.22 μm filters. Filtered material (HoFs) was diluted at the indicated concentration with distilled sterile water. In order

Abbreviations: HoFs, *Hanseniaspora opuntiae*-Filtrates; hpt, hours post treatment; hpi, hours post inoculation; PDA, potato dextrose agar media; YNB, yeast nitrogen base media.

to have a weight/volume concentration, the filtrated material (100%) was lyophilized and the concentration was determined (8.45 mg/ml).

In-Vitro Inhibitory Assay of *Corynespora cassiicola* and *Botrytis cinerea* Growth

Corynespora cassiicola growth and preparation of spore suspension were performed as previously described (Soares et al., 2009). *Botrytis cinerea* strain BMM was provided by Brigitte Mauch-Mani (University of Neuchatel, Switzerland). *Botrytis cinerea* growth and preparation of spore suspension were performed as previously described (L'Haridon et al., 2011). For the inhibitory assay, a spore suspension of *Corynespora cassiicola* (3×10^5 spores ml^{-1}) or *Botrytis cinerea* (5×10^4 spores ml^{-1}) was placed at the center of a Petri dish containing potato dextrose agar media (PDA) supplemented with 20, 30, 40, and 50% HoFs and incubated at 22°C for 72 h. Inhibition was evaluated by measuring the diameter of the mycelium on the dish. The experiment was carried out in a completely randomized design (CRD), with five replicates for each treatment. *Botrytis cinerea* spore germination assay was performed as previously described (Hael-Conrad et al., 2015). Pictures were taken at 24 hpi with a digital camera attached to a Leica DMR microscope with bright-field settings. Images of growing *Botrytis cinerea* hyphae were analyzed using Image J version 1.51 (NIH).

Plant Maintenance

Glycine max plants cultivar INT 6100, were grown under greenhouse conditions on pots containing non-autoclaved soil. *Arabidopsis thaliana* seeds were grown on a pasteurized soil mix of humus and perlite (3:1), kept at 4°C for 2 days and then transferred to the growth chamber. Plants were grown during 4 weeks in a 12 h light/12 h dark cycle with 60–70% of relative humidity, at a day temperature of 20–22°C and a night temperature of 16–18°C. *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK).

HoFs Treatment and *Corynespora cassiicola* or *Botrytis cinerea* Plant Inoculation

Corynespora cassiicola infection procedure and disease severity quantification were performed as previously described (Soares et al., 2009). *Glycine max* plants were grown until the V4 developmental stage (third fully expanded trifolium) and sprayed until saturation with 20% HoFs or mock (distilled sterile water) every 7 days, for 4 weeks. 24 h after the last treatment, plants were infected with a *Corynespora cassiicola* spore suspension (3×10^5 spores ml^{-1}) and 120 h post infection (hpi) disease severity was measured determining the minimum and maximum limits and the intermediate levels of the scale, according to Weber-Fechner's stimulus-response law, as previously described (Soares et al., 2009). *Botrytis cinerea* infection procedure and lesion size measurement were performed as previously described (L'Haridon et al., 2011). Four-week-old *Arabidopsis thaliana* plants were sprayed until saturation with 50% HoFs or mock (YNB) for 24, 48, 72, 96, or 120 h post treatment (hpt), as indicated in the Figure legends. After this time, 3 μl droplets containing *Botrytis cinerea* spore suspension (5×10^4 spores ml^{-1}) were applied. Infection symptoms were evaluated 72 hpi by measuring lesion size (cm). For the dose-response assay, plants were pre-treated with the indicated concentration HoFs and evaluated at 72 hpi. For the systemic assay, plants were pre-treated (watering the soil until saturation) with 50% HoFs or mock, and 24 hpt leaves were infected with *Botrytis cinerea* and evaluated at 72 hpi.

RNA Extraction

Arabidopsis thaliana leaves from 5 plants were harvested 24 hpt, pooled and immediately frozen in liquid nitrogen and kept at −80°C until use. Total RNA was extracted using the Spectrum™ Plant total RNA Kit (www.sigmaaldrich.com) as described in the manufacturer's protocols. The integrity of extracted RNA was measured by agarose gel electrophoresis (1.2%), concentrations and purity were determined by NanoDrop 2000/2000c (Thermo Fisher Scientific). Samples used for RNA-seq were also analyzed using an Agilent 2100 Bioanalyzer (Agilent Genomics).

Genome-Wide Transcriptomic Analysis

The RNA-seq libraries were prepared from isolated total RNA from 5 plants, pooled from three independent experiments, using the Illumina TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. The libraries were sequenced using an Illumina GAIIx platform for 72 paired-end cycles following the manufacturer's protocol. Sequences are publicly available through the Gene Expression Omnibus database under the accession number GSE113810 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=gse113810). Contamination and adapter removal was carried out using in-house Perl scripts. Fastq sequences were filtered based on quality (FASTQ Quality Filter v0.0.6, Q 33, http://hannonlab.cshl.edu/fastx_toolkit/index.html) and mapped on *Arabidopsis thaliana* transcriptome (TAIR10) using Bowtie2 (Langmead and Salzberg, 2012). Gene expression was calculated using RSEM v1.3 (Li and Dewey, 2011) and compared between the two RNA-seq libraries using DEGseq v3.6 (Wang et al., 2010), and the FPKM data from RSEM. Only transcripts with a Log2 fold change < -1 or > 1 with a p-value < 0.05 were considered. DEGs identified in by genome-wide transcriptomic analysis were analyzed and classified into gene ontology classes (GO) using the analysis toolkit agriGO (http://bioinfo.cau.edu.cn/agriGO/) previously described (Du et al., 2010). Identification of commonly regulated DEGs from previously published data and from the present work were performed using the software FiRe ver. 2.2 as previously described (Garcion and Metraux, 2006).

Real Time RT-PCR

Pooled total RNA (1.0 μg) from 5 plants, from two independent experiments, was retro-transcribed into cDNA according to the manufacturer's indications using the SCRIPT cDNA Synthesis Kit (Jena Bioscience www.jenabioscience.com). RT-qPCR was performed in 96-well plates with the Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific), using SYBR Green Maxima SYBR Green/ROX qPCR Master Mix (2X) (ThermoFisher Scientific,

www.thermofisher.com). Two independent experiments were analyzed with three technical replicates each. RT-qPCR conditions were as follows: an initial 95°C denaturation step for 15 min followed by denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C for 45 cycles. Gene expression values were normalized using the mean expression of two genes: AT4G26410 and AT1G72150 previously described as stable reference genes (Serrano and Guzmán, 2004; Czechowski et al., 2005). Normalized gene expression was determined using the comparative $2^{-\Delta\Delta CT}$ method previously described (Schmittgen and Livak, 2008). Primers for *ACS6*, *PR4*, and *PDF1.2* gene expression were previously described (Hael-Conrad et al., 2015).

RESULTS

Compounds Released by *Hanseniaspora opuntiae* Protect Against the Plant Pathogen *Corynespora cassiicola*

Yeasts have been characterized as biocontrol agents (BCAs) and eco-friendly alternatives to commercial pesticides against different plant pathogens (Liu et al., 2013); in particular, the antimicrobial compounds released, known as antifungal killer toxins or "mycocins" (Hatoum et al., 2012). In order to identify potential BCAs, a collection of yeast resident on *Theobroma cacao* fruits was isolated and the antimicrobial compounds released were tested against the fungal plant pathogen *Corynespora cassiicola* (Ferreira-Saab, 2018). One of the potential BCAs identified was *Hanseniaspora opuntiae,* which has been previously identified as part of the microbiome present in the cocoa bean fermentation process (Papalexandratou et al., 2013). In order to study the potential of *Hanseniaspora opuntiae* as biocontrol agent, *Corynespora cassiicola* spores were germinated on PDA media supplemented with 20% of compounds released by this yeast, identified as HoFs. *In-vitro* mycelia growth was inhibited by approximately 50%, compared to the PDA control media (**Figure 1A**). *Corynespora cassiicola* has been described as an important pathogen of many crop plants, including soybean (*Glycine max*). Then we determined if HoFs extended their biocontrol effect on this crop. Soybean plants were treated with 20% HoFs and after 24 hpt, infected with *Corynespora cassiicola* and at 120 hpi disease severity was quantified as previously described (Soares et al., 2009). A reduction of approximately 75% in disease severity, compared to the mock-treated control plants, was induced by HoFs 120 hpi (**Figure 1B**). These results indicated that HoFs not only inhibited *Corynespora cassiicola* growth *in-vitro*, but can be also used as BCAs on soybean plants.

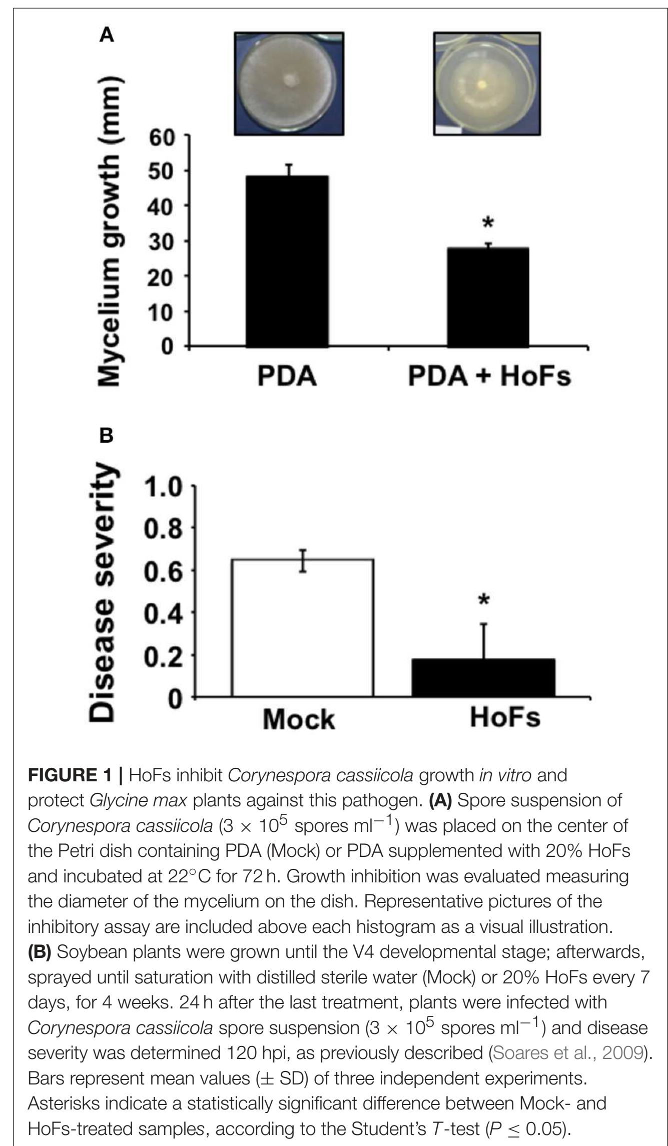

FIGURE 1 | HoFs inhibit *Corynespora cassiicola* growth *in vitro* and protect *Glycine max* plants against this pathogen. **(A)** Spore suspension of *Corynespora cassiicola* (3×10^5 spores ml^{-1}) was placed on the center of the Petri dish containing PDA (Mock) or PDA supplemented with 20% HoFs and incubated at 22°C for 72 h. Growth inhibition was evaluated measuring the diameter of the mycelium on the dish. Representative pictures of the inhibitory assay are included above each histogram as a visual illustration. **(B)** Soybean plants were grown until the V4 developmental stage; afterwards, sprayed until saturation with distilled sterile water (Mock) or 20% HoFs every 7 days, for 4 weeks. 24 h after the last treatment, plants were infected with *Corynespora cassiicola* spore suspension (3×10^5 spores ml^{-1}) and disease severity was determined 120 hpi, as previously described (Soares et al., 2009). Bars represent mean values (± SD) of three independent experiments. Asterisks indicate a statistically significant difference between Mock- and HoFs-treated samples, according to the Student's *T*-test ($P \leq 0.05$).

The Pathosystem *Arabidopsis thaliana-Botrytis cinerea* Can be Used as a Model to Analyze the HoFs-Induced Defense Mechanisms

In the field, application of BCAs has shown an inconsistent efficacy compared with synthetic chemical compounds and one possibility, to avoid this problem, is to better understand the molecular mechanisms behind the application of BCAs (Massart et al., 2015). In order to characterize the molecular mechanisms underlying the HoFs-induced biocontrol effect, we used the well-characterized plant-pathosystem *Arabidopsis thaliana–Botrytis cinerea*. First, we determined if HoFs inhibited the development of the necrotroph pathogen under *in-vitro* conditions (**Figure 2**). Analyzing a dose-dependent response, we observed that *Botrytis cinerea* grown on PDA media supplemented with 20 and 30% HoFs, showed about 25% inhibition of mycelial growth (**Figure 2A**). Increasing HoFs concentration up to 40 and 50%, directly correlated with a higher reduction of mycelial growth (between 70 and 80% inhibition, respectively), showing a dose-dependent response induced by HoFs (**Figure 2A**). To determine if HoFs directly affect the germination and the production of *Botrytis cinerea spores*, we analyzed the development of the fungus in the presence of 20% HoFs (**Figures 2B,C**). We determined that spores can germinate at 20% HoFs, but hyphae

growth was inhibited (**Figure 2B**). Additionally, we observed that mycelia developed under this conditions did not further produce spores (**Figure 2C**). These results suggest that HoFs have antifungal effect on *Botrytis cinerea.* Next, 4-week-old *Arabidopsis thaliana* plants were pre-treated with 50% HoFs 24 hpt and then infected with *Botrytis cinerea.* We observed a strong inhibition of the lesion caused by this pathogen on HoFs-treated plants compared to mock-treated samples, 72 hpi (**Figure 3A**). Additionally, a similar dose-dependent effect, observed under *in-vitro* conditions (**Figure 2A**), was determined *in planta,* since at higher HoFs concentration a smaller lesion size was quantified (**Figure 3B**). Then, to evaluate for how long HoFs can protect *Arabidopsis thaliana* plants against *Botrytis cinerea*, different hpt were assayed, measuring the lesion size at 72 hpi. For all of the times analyzed (24 to 120 hpt), HoFs-treated plants showed significant differences compared to mock-treated control samples (**Figure 4**), indicating that HoFs induced a protective effect over the plant-pathogen interaction at all of these time points. Taken together, these results indicated that HoFs protect *Arabidopsis thaliana* against *Botrytis cinerea* and that this pathosystem can be used as a model to characterize the molecular changes induced by HoFs application.

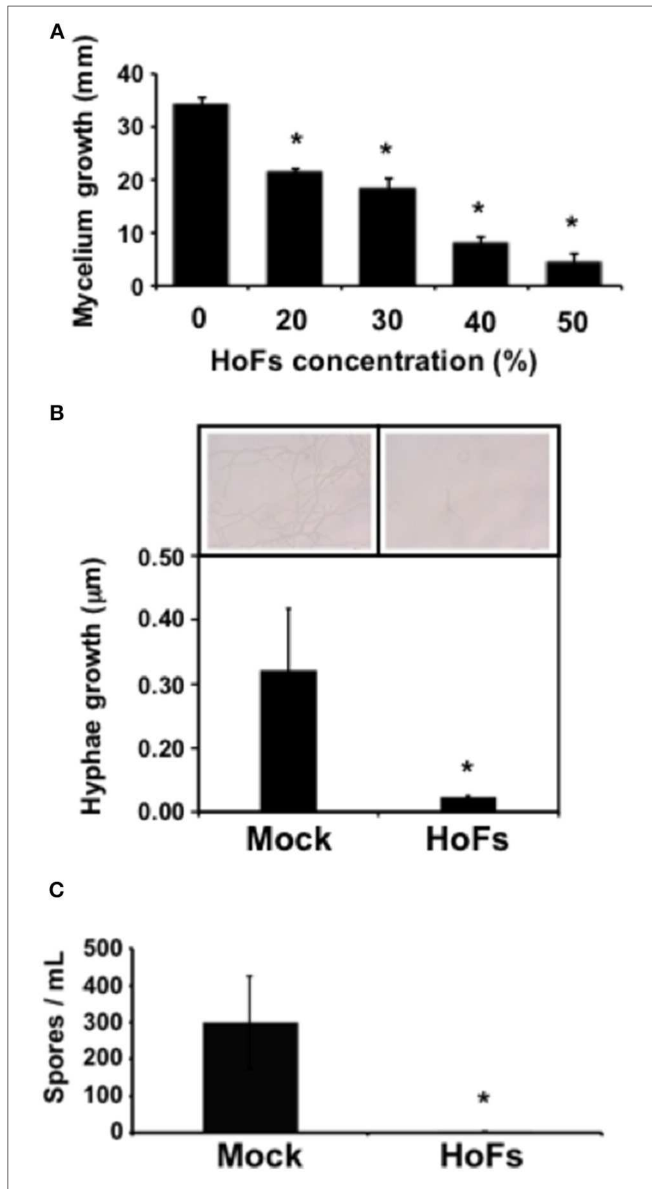

FIGURE 2 | HoFs induced a *Botrytis cinerea* development inhibition. **(A)** Spore suspension of *Botrytis cinerea* (5×10^4 spores ml^{-1}) was placed on the center of the Petri dish containing PDA supplemented with indicated concentrations of HoFs and incubated at 22°C. Growth inhibition was evaluated measuring the diameter of the mycelium on the dish 72 hpi. **(B)** Hyphae elongation produced by *Botrytis cinerea,* grown on 20% HoFs 24 hpi, was quantified as previously described (Hael-Conrad et al., 2015). A representative image of each treatment is presented. **(C)** Spores produced by *Botrytis cinerea* 15 days after the grown on 20% HoFs, were isolated and quantified as previously described (L'Haridon et al., 2011). Bars represent mean values (± SD) of three independent experiments. Asterisks indicate a statistically significant difference between 0% and the indicated concentrations of HoFs, according to the Student's *T*-test ($P \leq 0.05$).

HoFs Induced a Systemic Protection Against *Botrytis cinerea*

Under *in-vitro* conditions we observed an antifungal effect on *Botrytis cinerea* growth (**Figure 2**), this observation rises the

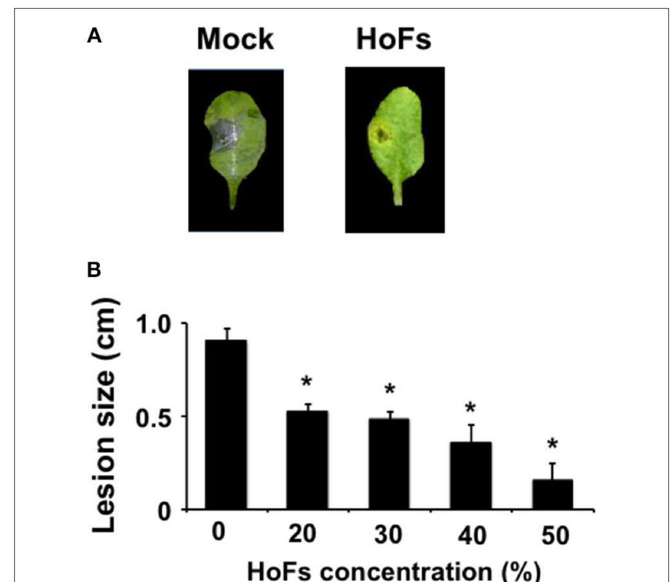

FIGURE 3 | HoFs protect *Arabidopsis thaliana* plants against *Botrytis cinerea.* **(A)** 4-week-old *Arabidopsis thaliana* plants were sprayed until saturation with YNB medium (Mock) or 50% HoFs. Twenty-Four hpt 3 μl droplets containing a *Botrytis cinerea* spore suspension (5×10^4 spores ml^{-1}) were applied and infection symptoms were evaluated 72 hpi. Representative pictures of the inhibitory assay are included as a visual illustration. **(B)** Four-week-old *Arabidopsis thaliana* plants were treated with the indicated HoFs concentration and infected as indicated above. Infection symptoms were evaluated 72 hpi by measuring lesion size (cm). Bars represent mean values (± SD) of three independent experiments each with twenty replicates. Asterisks indicate a statistically significant difference between Mock- and HoFs-treated samples, according to the Student's *T*-test ($P \leq 0.05$).

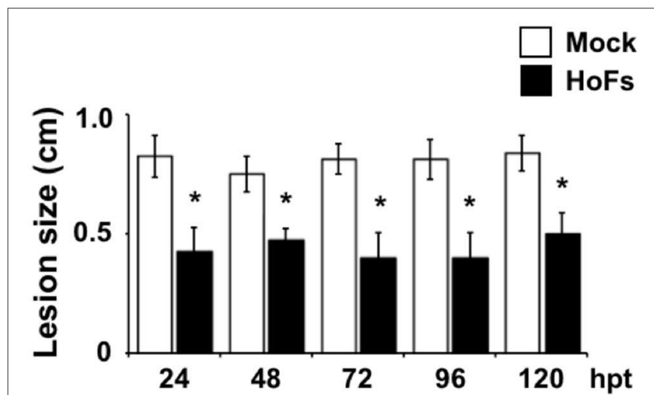

FIGURE 4 | Time-course growth inhibition of *Botrytis cinerea* in *Arabidopsis thaliana* plants treated with HoFs. 4-week-old *Arabidopsis thaliana* plants were sprayed until saturation with YNB medium (Mock) or 50% HoFs for 24, 48, 72, 96, and 120 (hpt), after these times 3 μl droplets containing *Botrytis cinerea* spore suspension (5×10^4 spores ml^{-1}) were applied. Infection symptoms were evaluated 72 hpi by measuring lesion size (cm). Bars represent mean values (± SD) of three independent experiments each with twenty replicates. Asterisks indicate a statistically significant difference between Mock- and HoFs-treated samples, according to the Student's *T*-test ($P \leq 0.05$).

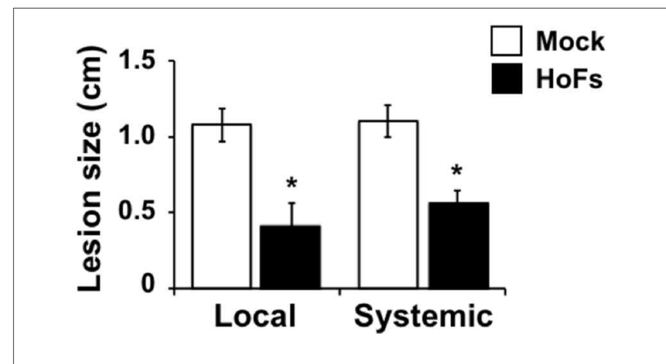

FIGURE 5 | Systemic effect induced by HoFs against *Botrytis cinerea* in *Arabidopsis thaliana* plants. 4 week-old *Arabidopsis thaliana* plants were pre-treated in the roots (watered soil until saturation) with YNB medium (Mock) or 50% HoFs and 24 hpt leaves infected with *Botrytis cinerea* and evaluated at 72 hpi. Bars represent mean values (± SD) of three independent experiments each with twenty replicates. Asterisks indicate a statistically significant difference between Mock- and HoFs-treated samples, according to the Student's *T*-test ($P \leq 0.05$).

questions of whether the protective effect observed *in planta* was induced by the direct effect of HoFs localized on the local leaf surface or by the modification of the plant defense responses itself. In order to clarify this question, we applied HoFs directly to the roots and we infected the untreated leaves (systemic) with *Botrytis cinerea*. 72 hpi HoFs-root-treated plants showed a similar significant reduction of lesion size, as the local HoFs-treated leaves (**Figure 5**). These results suggest two possibilities: (1) HoFs can be transported from the roots to the the entire plant, inhibiting *Botrytis cinerea* due to their antifungal effect and (2) HoFs might play a role as a potential elicitor of the defense responses that leads to a systemic resistance against the necrotrophic pathogen *Botrytis cinerea*. Either way, these result indicated that application of HoFs can triggered a systemic protection against this pathogen.

HoFs Induced a Reprograming of the *Arabidopsis thaliana* Transcriptome

During the last decade large-scale transcriptomic analysis have been used to understand how BCAs improve plant health (Massart et al., 2015). However, to our knowledge, only few a studies have used *Arabidopsis thaliana* as a model (Feng et al., 2012; Morán-Diez et al., 2012). In order to discover the transcriptional modifications induced by HoFs, the transcriptome of HoFs-treated plants was analyzed by RNA-seq (Supplementary Table 1, **Figure 6**). The expression of 186 and 46 genes was down- or up-regulated, respectively in HoFs-treated plants compared to non-induced samples (**Figure 6A**). GO analysis revealed that the most significant differentially expressed genes (DEGs), induced and repressed belonged to response to stress, chemical and abiotic stimulus, among others (**Table 1**).

GO analysis revealed that induced DEGs belonged to a group responsive to chitin, defense response, response to fungus and jasmonic acid (JA) biosynthetic processes (**Table 1B**). Previous reports have shown that *Arabidopsis thaliana* defense responses to *Botrytis cinerea* are JA- and ET-dependent (Thomma et al., 2001; Ferrari et al., 2003; Glazebrook, 2005). In order to validate the transcriptomic analysis, we compared the expression of JA- and ET-related genes, that were induced by HoFs (**Figure 6B**). Gene expression of the enzyme catalyzing the first and rate-limiting step of ET biosynthesis, 1-*aminocyclopropane-1-carboxylate synthase 6* (*ACS6*), the ET-responsive gene *PATHOGENESIS-RELATED 4* (*PR4*) and ET- and JA-responsive plant defensin gene (*PDF1.2*) were measured (**Figure 6B**). *ACS6*, *PR4* and *PDF1.2* have been previously described to be expressed during *Botrytis cinerea* infection (Windram et al., 2012; Hael-Conrad et al., 2015) and in agreement with these observations, we detected an up-regulation of these genes in HoFs-treated plants, compared to mock-treated samples (**Figure 6B**). These results help us to validate our genome-wide analysis and indicated that resistance to *Botrytis cinerea* induced by HoFs application, can be mediated, at least partially, by the transcriptional reprograming of the plant defense responses, in particular JA- and ET-induced pathway. This valuable information (Supplementary Table 1) can be used to uncover the HoFs-induced defense responses.

Transcriptional Reprograming Induced by HoFs Is Different Than Other BCAs Previously Reported

Two genome-wide transcriptomic analysis have been performed to characterize the mode of action of BCAs using *Arabidopsis thaliana* as a model. The first, analyzed the transcriptome changes induced by the pre-inoculation (24 hpi) of *Arabidopsis thaliana* plants with *Ralstonia solanacearum* ΔhrpB mutant strain, which has been previously shown to protect against the virulent strain of this phytopathogenic root bacteria on tomato (Frey et al.,

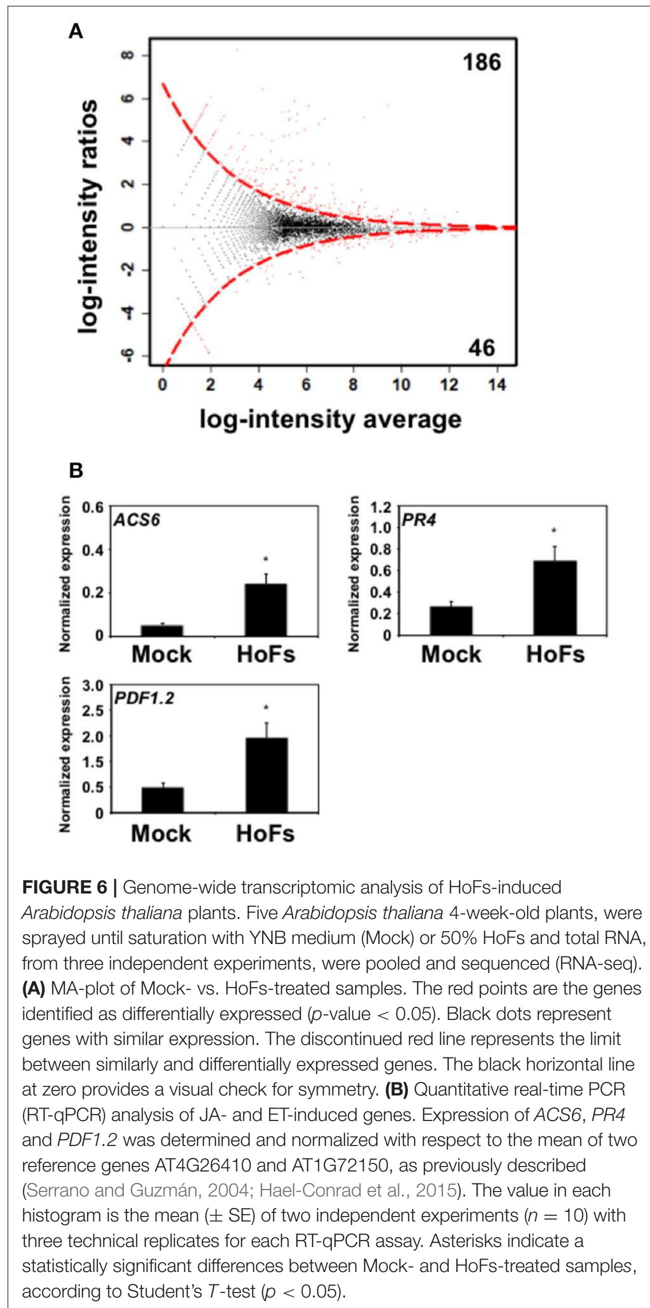

FIGURE 6 | Genome-wide transcriptomic analysis of HoFs-induced *Arabidopsis thaliana* plants. Five *Arabidopsis thaliana* 4-week-old plants, were sprayed until saturation with YNB medium (Mock) or 50% HoFs and total RNA, from three independent experiments, were pooled and sequenced (RNA-seq). **(A)** MA-plot of Mock- vs. HoFs-treated samples. The red points are the genes identified as differentially expressed (p-value < 0.05). Black dots represent genes with similar expression. The discontinued red line represents the limit between similarly and differentially expressed genes. The black horizontal line at zero provides a visual check for symmetry. **(B)** Quantitative real-time PCR (RT-qPCR) analysis of JA- and ET-induced genes. Expression of *ACS6*, *PR4* and *PDF1.2* was determined and normalized with respect to the mean of two reference genes AT4G26410 and AT1G72150, as previously described (Serrano and Guzmán, 2004; Hael-Conrad et al., 2015). The value in each histogram is the mean (± SE) of two independent experiments ($n = 10$) with three technical replicates for each RT-qPCR assay. Asterisks indicate a statistically significant differences between Mock- and HoFs-treated samples, according to Student's T-test ($p < 0.05$).

1994). From this analysis 152 and 336 genes were identified to be down- and up-regulated, respectively (Feng et al., 2012). Interestingly, 26% of the up-regulated genes were related to biosynthesis of abscisic acid (ABA) and signaling, suggesting an important role of this plant hormone on the defense mechanisms induced by this BCA (Feng et al., 2012). The other, described the transcriptomic response of *Arabidopsis thaliana* plants after inoculation with the biocontrol fungus *Trichoderma harzianum* at 24 hpi (Morán-Diez et al., 2012). From this analysis, only 66 DEGs were identified, 33 up- and 33 down-regulated as a result of the interaction (Morán-Diez et al., 2012). The expression of SA- and JA-related genes was down regulated, while genes involved in the abiotic stresses were induced (Morán-Diez et al., 2012). Here, in order to identify if treatments with BCAs share a similar transcriptomic signature, we analyzed the commonly co-expressed DEGs in *Arabidopsis thaliana* plants treated for 24 hpi with *Ralstonia solanacearum* ΔhrpB mutant strain, 24 hpi with *Trichoderma harzianum* and 24 hpt with HoFs (**Figure 7**). Only 2 and 7 genes were down- and up-regulated, respectively, after the pre-inoculation with ΔhrpB mutant and infection with *Trichoderma harzianum* (**Figure 7**), indicating that the two biocontrols triggered different defense response pathways. However, is worth to mentioning that we determined that DEGs induced or repressed by HoFs are not part of the same core of genes regulated by these other BCAs (**Figure 7**). These results indicate that HoFs-induced DEGs have not been previously identified as part of BCAs-induced defense mechanisms.

DISCUSSION

HoFs Have the Potential to Protect Against the Broad Host-Range Necrotrophic Fungi *Corynespora cassiicola* and *Botrytis cinerea*

The necrotrophic fungi *Corynespora cassiicola* and *Botrytis cinerea* are considered as important plant pathogens that affect pre- and post-harvest processes. *Corynespora cassiicola* resides on plant surfaces, nematodes cysts and human skin and can infect at least 530 plant species, including several important crops such as cowpea, cucumber, papaya, rubber, soybean and tomato (Dixon et al., 2009). While *Botrytis cinerea,* is a broad host-range necrotrophic fungus, commonly known as gray mold, that can infect more than 200 plant species, and for this, it has been classified as the second most important phytopathogen (Dean et al., 2012). Several elicitors have been previously described to protect the plants against *Botrytis cinerea*, including rhamnolipids, oligogalacturonides, chitosan, ceratoplatanin and the proteins PebC1 and AsES (Trotel-Aziz et al., 2006; Ferrari et al., 2007; Sanchez et al., 2012; Baccelli et al., 2014; Zhang et al., 2014; Feng et al., 2015; Hael-Conrad et al., 2015). However, to our knowledge, there is only one report where biocontrol agents were analyzed for their effect against *Corynespora cassiicola* under *in-vitro* and in field conditions. This early study, included the microorganisms *Trichoderma spp*., *Bacillus subtilis*, and *Pseudomonas florescence* and the elicitors from garlic bulb and neem seed kernel extracts (Manju et al., 2014). In our work, we determined that the elicitors released by the biocontrol yeast *Hanseniaspora opuntiae* (HoFs) can protect *Glycine max* and *Arabidopsis thaliana* plants against the necrotroph pathogens *Corynespora cassiicola* and *Botrytis cinerea,* respectively. Under *in-vitro* and *in-planta* conditions, HoFs show a dose-dependent behavior, similar to other elicitors previously characterized (Trotel-Aziz et al., 2006; Hael-Conrad et al., 2015). Additionally, we determined that the HoFs-induced protective effect on *Arabidopsis thaliana* plants against *Botrytis cinerea*, can be induced after 24 h pretreatment and maintained without

TABLE 1 | Gene ontology (GO) enrichment analysis of differentially expressed genes of HoFs-treated *Arabidopsis thaliana* plants.

GO ID	Description	No. Genes	*p*-Value
(A)			
GO:0009628	Response to abiotic stimulus	9	5.90E-06
GO:0050896	Response to stimulus	14	7.10E-06
GO:0006950	Response to stress	11	5.00E-06
GO:0042221	Response to chemical stimulus	10	1.40E-05
GO:0006810	Transport	7	1.30E-03
GO:0051234	Establishment of localization	7	1.40E-03
GO:0051179	Localization	7	1.70E-03
GO:0009725	Response to hormone stimulus	5	2.00E-03
GO:0009719	Response to endogenous stimulus	5	2.90E-03
GO:0010033	Response to organic substance	5	7.60E-03
GO:0022891	Substrate-specific transmembrane transporter activity	5	1.40E-03
GO:0022892	Substrate-specific transporter activity	5	2.80E-03
GO:0022857	Transmembrane transporter activity	5	3.70E-03
GO:0005215	Transporter activity	5	1.10E-02
(B)			
GO:0006950	Response to stress	51	1.10E-23
GO:0050896	Response to stimulus	61	2.50E-20
GO:0015979	Photosynthesis	16	3.80E-17
GO:0042221	Response to chemical stimulus	41	3.60E-17
GO:0006091	Generation of precursor metabolites and energy	18	5.90E-16
GO:0009611	Response to wounding	14	2.60E-13
GO:0010033	Response to organic substance	29	3.30E-13
GO:0009605	Response to external stimulus	18	4.70E-13
GO:0044237	Cellular metabolic process	77	5.90E-13
GO:0019684	Photosynthesis, light reaction	11	1.70E-12
GO:0010200	Response to chitin	12	4.00E-12
GO:0009987	Cellular process	89	9.70E-12
GO:0008152	Metabolic process	83	2.80E-11
GO:0009409	Response to cold	14	1.70E-10
GO:0009743	Response to carbohydrate stimulus	12	6.40E-10
GO:0009607	Response to biotic stimulus	17	1.90E-09
GO:0009266	Response to temperature stimulus	15	2.60E-09
GO:0044249	Cellular biosynthetic process	48	5.60E-09
GO:0031408	Oxylipin biosynthetic process	6	1.00E-08
GO:0009628	Response to abiotic stimulus	24	1.10E-08
GO:0009145	Purine nucleoside triphosphate biosynthetic process	7	1.50E-08
GO:0009142	Nucleoside triphosphate biosynthetic process	7	1.60E-08
GO:0009144	Purine nucleoside triphosphate metabolic process	7	1.60E-08
GO:0009141	Nucleoside triphosphate metabolic process	7	2.00E-08
GO:0009058	Biosynthetic process	48	1.90E-08
GO:0031407	Oxylipin metabolic process	6	3.00E-08
GO:0009620	Response to fungus	9	3.30E-08
GO:0051707	Response to other organism	15	3.90E-08
GO:0009414	Response to water deprivation	10	6.00E-08
GO:0009150	Purine ribonucleotide metabolic process	7	7.40E-08
GO:0009415	Response to water	10	9.20E-08
GO:0006164	Purine nucleotide biosynthetic process	7	1.60E-07
GO:0006952	Defense response	16	1.50E-07
GO:0006163	Purine nucleotide metabolic process	7	1.80E-07
GO:0006970	Response to osmotic stress	12	1.80E-07
GO:0009259	Ribonucleotide metabolic process	7	2.40E-07
GO:0009695	Jasmonic acid biosynthetic process	5	2.70E-07
GO:0015992	Proton transport	6	2.90E-07
GO:0006818	Hydrogen transport	6	2.90E-07

(Continued)

*Biological process identified to be **(A)** down-regulated and **(B)** up-regulated on HoFs-treated compared to Mock-treated samples.*

significant reduction for up to 5 days (**Figure 4**). Taken together, these results indicated that HoFs have the potential to be used as biocontrols against these agronomically important pathogens. Furthermore, it will be interesting to study if HoFs can protect against other pathogens, including other fungi, bacteria and/or herbivores.

HoFs Induce Local and Systemic Protection Against *Botrytis cinerea*

HoFs show a protective effect *in-planta*, but additionally, they also inhibited the development of the pathogens under *in-vitro* conditions (**Figures 1**, **2**). These results suggest that HoFs might work as fungicides, however, since we also observed a systemic protection in *Arabidopsis thaliana* plants (**Figure 5**), we can not discard the idea that HoFs can either be diffused through the whole plant and/or that, once inside the plant cell, they can induce the defense responses as true elicitors. The possibility that HoFs might act as elicitors inducing the defense responses is supported by the changes in the genome-wide transcriptomic machinery, since genes of the JA- and ET-related pathways that have been previously reported to be involved in the *Botrytis cinerea* response, are induced (**Figure 6**). Interestingly, a similar *in-vitro* inhibitory effect on *Botrytis cinerea* and the induction of the defense responses have been observed with other well-characterized elicitor, the chitosan (Trotel-Aziz et al., 2006). Exogenous application of elicitors has diverse and, sometimes, contradictory effect. While chitosan has been described to improve plant growth (Yin et al., 2016), constitutive activation of the defense responses by oligogalacturonides (OGs), have been recently shown to affect the plant growth rate, suggesting a defense-growth trade-off (Benedetti et al., 2018). Now, the question if HoFs have a similar effect is still open. Either way, the local and systemic protection induced by HoFs, might facilitate their application and might give them the potential to be used on the field to protect the crops against these pathogens.

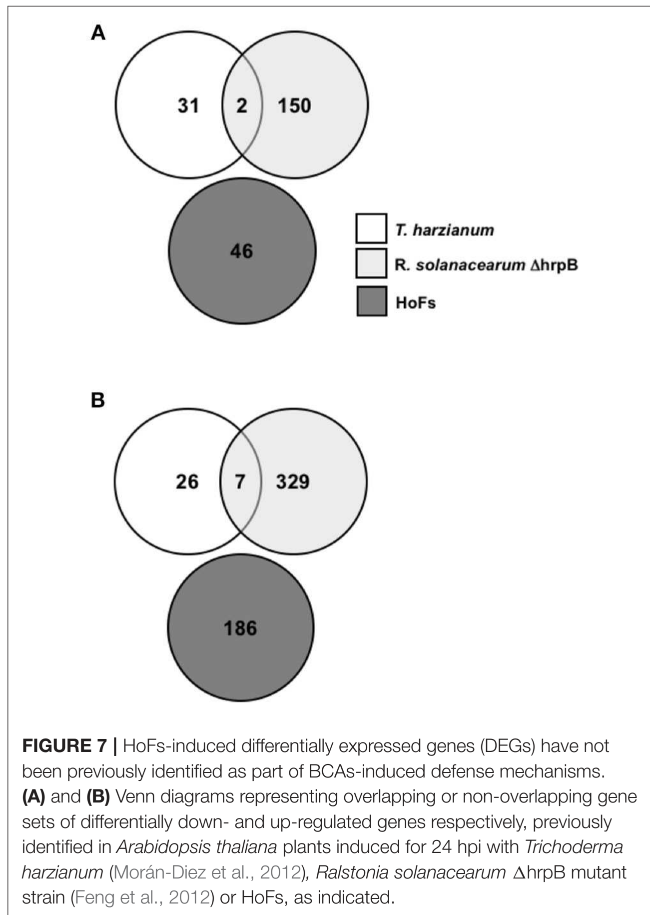

FIGURE 7 | HoFs-induced differentially expressed genes (DEGs) have not been previously identified as part of BCAs-induced defense mechanisms. **(A)** and **(B)** Venn diagrams representing overlapping or non-overlapping gene sets of differentially down- and up-regulated genes respectively, previously identified in *Arabidopsis thaliana* plants induced for 24 hpi with *Trichoderma harzianum* (Morán-Diez et al., 2012), *Ralstonia solanacearum* ΔhrpB mutant strain (Feng et al., 2012) or HoFs, as indicated.

HoFs Might Induce Systemic Protection Against *Botrytis cinerea* by Triggering JA- and ET-Dependent Signaling Pathways, but Not SA-Induced Pathway

In order to regulate the complex interactions with the microorganisms, plants have developed inducible defense responses. The first line of defense, that is induced by the recognition of molecules, including the elicitors, is called plant innate immunity (Boller and Felix, 2009). Once the immunity is induced, the response is amplified by the induction of SA-, JA-, and ET-induced signaling pathways (Garcion et al., 2007; Dangl et al., 2013). These defense mechanisms work coordinately to regulate the plant-pathogen interactions, locally and systemically by priming the defense responses, including the systemic and induced acquired resistance (SAR and IAR) (Craig et al., 2009; Tsuda and Somssich, 2015). Here we proposed the possibility that HoFs might work as elicitor to induce a systemic protection against *Botrytis cinerea* (**Figure 5**). JA- and ET-related genes are induced after HoFs application (**Figure 6**, **Table 1**), but the SA-induced gene *PR1* is actually repressed (Supplementary Table 1). For decades, SA has been proposed to govern the induction of SAR, however, multiple reports have revealed that systemic defense responses are not regulated and induced only by SA but by an intricate and complex network that involves other phytohormones including JA and ET (reviewed by Conrath et al., 2015; Klessig et al., 2018). With this in mind, characterization of HoFs-induced defense responses warrants further studies.

Exploring the Pathosystem *Arabidopsis thaliana-Botrytis cinerea* to Characterize HoFs-Induced Defense Mechanisms

Elicitors have the potential to be used in agriculture as an alternative to chemical fungicides, however, in order to optimize their application and activity on the field, it is necessary to know and characterize their mode of action (Wiesel et al., 2014). In this report, we used the well characterized pathosystem *Botrytis cinerea-Arabidopsis thaliana* to identify the transcriptomic changes induced by HoFs (**Figure 6**, **Table 1,** Supplementary Table 1). Using genetic, molecular and *omics* tools applied on different plant models, including *Arabidopsis thaliana*, plant-microbe and microbe-microbe interactions, have been characterized at the molecular level (Kroll et al., 2017). In the plants, this characterization includes, the analysis of the early events during the beneficial and pathogenic interactions (Zipfel and Oldroyd, 2017), the transcriptional regulation of plant defense responses (Birkenbihl et al., 2017) and the elicitor-mediated activation of plant immunity (Cheng et al., 2018). On the other hand, the molecular analysis of the pathogens *Corynespora cassiicola* and *Botrytis cinerea* also has also been improved with the identification of the genomic sequence and the transcriptomic characterization during the interaction with the plants (Windram et al., 2012; Shrestha et al., 2017; Van Kan et al., 2017). Now, with all this available information and with the HoFs-induced DEGs identified from our work, further studies are warranted, that might help us to understand the molecular defense mechanisms induced by HoFs.

Triggered Transcriptional Modulation of Plant Defense Responses Is Broadly BCAs-Specific

Only two BCAs have been characterized by analyzing genome-wide transcriptional changes in *Arabidopsis thaliana*, using the bacterium *Ralstonia solanacearum* ΔhrpB mutant strain and the fungus *Trichoderma harzianum* (Feng et al., 2012; Morán-Diez et al., 2012). In order to identify similarities between the transcriptome induced by different-origin BCAs, we compared the DEGs from these two reports and those induced by yeast-derived HoFs (**Figure 7**). Remarkably, we observed that only 9 DEGs are shared in response to *Trichoderma harzianum* and *Ralstonia solanacearum* treatments and that there were no similarities with HoFs treatment (**Figure 7**). In agreement with these observations, it was previously reported that the expression of JA-related genes was down-regulated after *Ralstonia solanacearum* induction (Morán-Diez et al., 2012), while we determined that after HoFs treatment these genes were up-regulated (**Figure 6B**). Similar differential responses have been described in others plant-microbe interactions, for example, the pathogenic bacterium *Pseudomonas syringae* has been shown to induced the SA-induced signaling pathway (Grant and Jones, 2009; Verhage et al., 2010), while the fungus *Botrytis*

cinerea induced JA- and ET-signaling pathways (Thomma et al., 2001; Glazebrook, 2005). To further highlight the complexity of these interactions, other reports have also shown contradictory results on the phytohormone-dependent responses induced by biotrophic and necrotrophic pathogens, since complex cross-talks and multifactorial dependence between SA-, JA-, and ET-signaling pathways have been described (Koornneef and Pieterse, 2008; Pieterse et al., 2009; Hael-Conrad et al., 2015). These observations suggest that both, the triggered defense mechanisms and the protective effect against a particular pathogen(s) are differentially regulated depending of the origin of BCAs.

Summarizing, HoFs induce local and systemic defense responses to broad host-range necrotrophic fungi. HoFs induce a transcriptional reprograming of *Arabidopsis thaliana* plants, and this genome-wide information can be used as starting point to understand the molecular basis of HoFs-triggered responses. Future work is now directed to characterize the biochemical nature of HoFs, including the chemical identity/identities of the elicitor(s).

AUTHOR CONTRIBUTIONS

DF, CS, AT, KS-E and MS conceived and designed the experiments. MF-S, MT, WA, EP, and DF performed the experiments. DF, CS, AT, and MS wrote and revised the paper. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01596/full#supplementary-material

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