TESIS DE DOCTORADO

PEDECIBA

Área Biología

Cancro del tallo de la soja: Caracterización molecular del patosistema *Glycine max-Diaporthe phaseolorum*

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RESUMEN

En Uruguay la soja (*Glycine Max* L. Merr) constituye el principal producto de exportación. Sus plantaciones son afectadas por enfermedades como el cancro del tallo que afectan la calidad del grano y disminuye su producción. La misma es causada por especies del género *Diaporthe,* incluyendo *D. aspalathi, D. caulivora* y *D. longicolla*. *D. caulivora* es la especie predominante en Uruguay, la cual al colonizar los tejidos produce síntomas que comienzan como pequeñas lesiones en el tallo que se expanden a lo largo llegando a causar la muerte de la planta.

El objetivo del proyecto de tesis fue caracterizar la interacción de la soja con el hongo *Diaporthe caulivora*. Para ello se analizó la variabilidad genética de 78 aislados del complejo *Diaporthe/Phomopsis* obtenidos de lesiones de cancro de diferentes zonas del país, siendo *D. caulivora* y *D. longicolla* las especies predominantes. Se seleccionó uno de los aislados de *D. caulivora* y se inocularon plantas de soja susceptibles, las cuales desarrollaron los síntomas característicos de la enfermedad, observándose clorosis y necrosis en el sitio de infección cinco días posteriores a la inoculación. Las lesiones avanzaron de forma progresiva en ambas direcciones del tallo hasta alcanzar casi la totalidad de la planta. Posteriormente, se compararon tres aislados de *D. caulivora* y se observaron diferencias en el proceso de infección, siendo D57 el más agresivo y D08.4 el menos agresivo. Se evidenció la colonización del tejido vegetal del aislado virulento D57, con hifas asociadas a los tricomas como potencial punto de entrada y la proliferación del hongo dentro de los tejidos, ocupando haces vasculares. La cuantificación de la biomasa del patógeno evidenció un aumento a partir de las 8 horas posteriores a la inoculación (hpi), llegando a representar el 50% de la muestra a las 96 hpi.

En respuesta a la infección con *D. caulivora*, las plantas de soja activaron mecanismos de defensa que se evidenciaron por una fortificación de la pared celular en el área infectada dada por la acumulación de compuestos fenólicos. Se observó además un aumento en los niveles de expresión de genes de defensa, incluyendo PR-1, PR-2, PR-3, PR-4, PR-10, IPER, PAL, CHS y LOX en plantas inoculadas con *D. caulivora*.

Por otro lado, se secuenció, ensambló y analizó por primera vez el genoma de *D. caulivora* (aislado D57) y se estudió mediante RNA-seq el perfil transcriptómico de *D. caulivora* durante la colonización de la planta. El tamaño estimado del genoma es de 57,86 Mb y contiene 18.385 genes codificantes. Se identificaron genes relacionados con la patogenicidad de *D. caulivora*, tales como enzimas de hidrato de carbono-activas (CAZymes), oxidorreductasas, poliquétido sintasas y efectores los cuales interfieren con los mecanismos de defensa de la planta. Los datos del transcriptoma de *D. caulivora* crecido en placas de cultivo con medio PDA y en plantas de soja inoculadas a las 8 y 48 hpi evidenciaron una expresión diferencial de 2659 genes de *D. caulivora*. Los patrones de expresión de genes regulados positivamente y el enriquecimiento de genes revelaron que las estrategias de infección de este patógeno están relacionadas con la degradación y modificación de la pared celular de la planta, la inducción de necrosis, las actividades de transporte y la producción de toxinas. El análisis combinado del genoma y transcriptoma brinda nueva e importante información sobre los mecanismos moleculares implicados en la patogénesis de *D. caulivora* y el proceso de colonización del hospedero.

Con el objetivo de tener más información sobre los mecanismos de defensa activados por las plantas de soja luego de la infección con *D. caulivora*, se compararon dos cultivares contrastantes de soja, Williams y Genesis 5601, frente a la infección con *D. caulivora* en condiciones controladas. *D. caulivora* causa enfermedad en ambos cultivares, observándose mayor largo de las lesiones, severidad de la enfermedad, y biomasa del patógeno en el cultivar Williams susceptible. Por último, se comparó la expresión diferencial de genes mediante RNA-seq de ambos cultivares inoculados con *D. caulivora* o control (0, 8 y 48 hpi). Los transcriptomas revelaron diferentes patrones de expresión entre plantas inoculadas respecto a sus controles y también entre cultivares. En total se expresan diferencialmente 1855 y 2322 genes en Williams y Genesis 5601, respectivamente. A tiempo cero se expresan diferencialmente 164 genes entre ambos cultivares. En Genesis 5601 se sobreexpresan 73 genes entre los que se encuentran receptores PRRs y genes relacionados con la defensa vegetal. Además, se observó una respuesta de defensa más rápida en el cultivar resistente, detectándose a 8 las hpi 1028 genes sobreexpresados en Genesis 5601 y solo 434 genes en Williams. Los patrones de expresión de genes regulados positivamente y el análisis de enriquecimiento de ontología mostraron que en la activación de defensa vegetal juegan un rol importante las vías hormonales y la ruta de los fenilpropanoides. La respuesta temprana de Genesis 5601 a la infección con *D. caulivora* así como las diferencias basales respecto al cultivar Williams es consistente con el grado de resistencia/susceptibilidad observado en nuestro trabajo. Los resultados obtenidos en esta tesis constituyen aportes originales sobre este patosistema y brindan información relevante sobre las bases moleculares de la interacción soja-*D. caulivora,* los cuales pueden ser utilizados en los programas de mejoramiento de la soja.

INTRODUCCIÓN

Glycine max

La soja (*Glycine Max* L. Merr) es una leguminosa de ciclo anual que pertenece a la familia de las papilonáceas (fabáceas). La soja tiene su origen en el norte y centro de China (Ridner, 2006). En la India se consumió a partir de 1735 y en Francia y otros países de Europa se plantaron las primeras semillas en 1740. Posteriormente, en 1765 se introdujo en el continente americano, en Georgia, Estados Unidos. Los japoneses la incorporaron después de la guerra chino-japonesa (1894-1895) y comenzaron a importar tortas de aceite de soja para usarlas como fertilizantes. La expansión a gran escala de la soja se efectuó a partir de la cuarta década del siglo XX (Ridner, 2006; Qiu and Chang, 2010).
Las plantas presentan porte erguido, alcanzando entre 0,50 y 1,5 metros de altura. Posee hojas grandes, trifoliadas y pubescentes. Sus flores son pequeñas, de color blanco-amarillento o azul-violáceo, se agrupan en inflorescencias y se ubican en las axilas de las hojas. Las vainas son cortas y contienen en su interior entre uno y cuatro granos oleaginosos, con distintas variaciones de color de la testa. La forma de la semilla es variable, desde esférica hasta ovalada y el peso de 100 semillas puede ser desde 5 hasta 40 g dependiendo del cultivar utilizado (Ridner, 2006; Rosas y Young 1991).
El cultivo de la soja requiere abundante agua y humedad relativa de 50% en las primeras etapas del desarrollo. Durante la floración, la formación de vainas y el llenado del fruto requiere agua disponible, ya que un estrés hídrico se refleja en el aborto de estructuras reproductoras y en el tamaño de las semillas (Rosas y Young 1991). Por otro lado, la soja es una planta de días cortos, pero hay una considerable variación genética para la sensibilidad al fotoperíodo. Su cultivo tiene un rango de temperatura óptima de 22 a 30°C (Rosas y Young 1991).
Existen varias clasificaciones para identificar los distintos estados de desarrollo en soja, la más difundida es la escala desarrollada por Fehr *et al.* (1971), donde se describe los estadios fenológicos externos del cultivo de soja, distinguiéndose dos etapas principales; los estados vegetativos y los reproductivos que describimos a continuación:

Etapas vegetativas:

VE- Emergencia: Se observa el hipocótilo, en forma de arco, empujando al epicótile y a los cotiledones, haciéndolos emerger sobre la superficie del suelo.

VC- Etapa cotiledonar: El hipocótilo se endereza, los cotiledones se despliegan totalmente y en el nudo inmediato superior los bordes de las hojas unifoliadas no se tocan.

V1- (1er nudo): El par de hojas opuestas unifoliadas están expandida totalmente, y en el nudo inmediato superior se observa que los bordes de cada uno de los foliolos de la 1er hoja trifoliada no se tocan.

V2- (2do nudo): La 1er hoja trifoliada está totalmente desplegada, y en el nudo inmediato superior los bordes de cada uno de los foliolos de la 2da hoja trifoliada no se están tocando.

Vn- (n: número de nudos): La hoja trifoliada del nudo (n) está expandida totalmente, y en el nudo inmediato superior los bordes de cada uno de los foliolos no se tocan.

Etapas reproductivas

R1- Inicio de Floración: Se observa una flor abierta en cualquier nudo del tallo principal.

R2- Floración completa: Se observa una flor abierta en uno de los nudos superiores del tallo principal con hojas totalmente desplegadas.

R3- Inicio de formación de vainas: Una vaina de 5 mm de largo en uno de los 4 nudos superiores del tallo principal, y con hojas totalmente desplegadas.
R4- Vainas completamente desarrolladas: Una vaina de 2 cm en uno de los 4 nudos superiores del tallo principal con hojas totalmente desplegadas. En esta etapa comienza el período crítico del cultivo (R4,5-R5,5) donde cualquier situación de stress: déficit hídrico, de nutrientes, defoliación por orugas, enfermedades foliares, ataque de chinches, granizo, etc, afectará el número final de vainas y de granos, provocando la reducción del rendimiento.
R5- Inicio de formación de semillas: Una vaina, ubicada en uno de los 4 nudos superiores del tallo principal, contiene una semilla de 3 mm de largo.
R6- Semilla completamente desarrollada: Una vaina, en cualquiera de los cuatro nudos superiores del tallo principal, contiene una semilla verde que llena la cavidad de dicha vaina, con hojas totalmente desplegadas. En esta etapa termina el período crítico del cultivo.
R7- Inicio de maduración: Una vaina normal en cualquier nudo del tallo principal ha alcanzado su color de madurez. La semilla, en este momento, contiene el 60% de humedad.
R8- Maduración completa: El 95% de las vainas de la planta han alcanzado el color de madurez. Luego de R8, se necesitan cinco a diez días de tiempo seco (baja humedad relativa ambiente), para que las semillas reduzcan su humedad por debajo del 15%.

La importancia de la soja está dada principalmente por ser una rica fuente de proteínas y aceite que aporta un alto valor nutricional (He y Chen 2013). La composición del grano es en promedio; 36,5% de proteínas, 20% de lípidos, 30% de hidratos, 8,5% de agua y 5% de otros compuestos (Ridner, 2006). El aceite de soja es rico en ácidos grasos (22% oleico, 54% linoleico, 10% palmitico, 4% estearico y 10% linolenico), por lo que es considerado uno de los aceites vegetales más saludables disponibles para el consumo humano (Wilson, 2004). La soja contiene todos los aminoácidos esenciales en cantidad suficiente para satisfacer los requerimientos nutricionales, incluyendo los sulfurados (metionina y cisteína) a diferencia de otros alimentos de origen vegetal (Rosas y Young, 1991). Además, contiene una amplia gama de minerales como el calcio, hierro, cobre, fósforo y zinc y vitaminas como tiamina (B1), riboflavina (B2), piridoxina (B6), niacina, ácido pantoténico, biotina, ácido fólico, β-caroteno (provitamina A), ácido ascórbico (vitamina C), inositol y colina. Además, posee tocoferoles, que constituyen antioxidantes naturales y tienen funciones de vitamina E (Ridner, 2006).
La producción en Uruguay en el 2019/2020 fue de 2.828.000 toneladas con rendimiento de 2.928 Kg/Ha para 966.000 Ha cultivadas. A nivel mundial la producción fue de 333.671.692 toneladas con un rendimiento de 2.769 Kg/Ha en un área de 120.501.628 Ha. Uruguay se encuentra ubicado entre dos grandes productores y exportadores de soja a nivel mundial: Brasil y Argentina (FAO, 2021).
Los factores que limitan la productividad de la soja están relacionados, entre otros, con el manejo de suelo-cultivo, condiciones de ambiente desfavorables incluyendo principalemente la sequía y con factores bióticos, como las malezas, plagas y enfermedades (Ivancovich y Botta, 2003).

Principales enfermedades de la soja

Existen muchas enfermedades del cultivo de la soja, algunas de las cuales causan pérdidas significativas en la producción. La severidad de los daños y la aparición de enfermedades dependen de las condiciones ambientales, especialmente la temperatura y la humedad relativa, la susceptibilidad de las variedades de soja y la virulencia de los patógenos (Rosas y Young 1991). Se han descrito más de 135 patógenos en soja y se considera que alrededor de 30 especies pueden causar daños económicamente significativos (Roy *et al.* 2000), siendo los hongos los más perjudiciales (Vidić *et al*., 2013).

Muchas enfermedades atacan el cultivo de la soja en Uruguay. Para su estudio se dividen en tres grupos dependiendo del órgano al que infectan: hoja, tallo y raíz. Las hojas son afectadas por hongos que provocan enfermedades como la mancha marrón o septoriosis (*Septoria glycines* Hemmi), el tizón de la hoja y mancha púrpura de la semilla (*Cercospora kikuchii* (Matsumoto & Tomoy) Gardner), la mancha en ojo de rana (*C. sojina* Hara), oídio (*Microsphaera diffusa* Cooke & Peck), mildiú (*Peronospora manshurica (*Naumov) Syd) y la roya asiática (*Phakopsora pachyrhizi* Syd. & Syd.). Además, las bacterias causan el tizón bacteriano (*Pseudomonas savastanoi* pv. *glycinea* (Coerper) y la pústula bacteriana (*Xanthomonas axonopodis* pv. *glycines* (Nakano). Otras enfermedades causadas por virus son el mosaico común de la soja y el mosaico de la alfalfa (Rosas y Young 1991; Stewart y Rodríguez, 2013).

Por otro lado, las afecciones de tallo y raíz son producto principalmente de la infección con hongos. En tallo existen enfermedades como el cancro del tallo, causado por dos variedades del patógeno *Diaporthe caulivora (syn. Diaporthe phaseolorum* var. *caulivora* (Athow & Caldwell) y *Diaporthe aspalathi (syn. Diaporthe phaseolorum* var. *meridionalis* (Fernández & Hanlin) y recientemente se sumó *Diaporthe longicolla (syn. Phomopsis longicolla)* (Mathew *et al*., 2015). Además, otras enfermedades como el tizón del tallo y de la vaina (*D. phaseolarum* var. *sojae*), la antracnosis (*Colletotrichum truncatum* (Schwein.) Andrus & Moore, *Colletotrichum destructivum* O'Gara, *Colletotrichum gloesporioides* (Penz.) Penz. & Sacc y *Colletotrichum graminícola* (Ces.) G.W. Wilson), el moho blanco o podredumbre húmeda (*Sclerotinia sclerotiorum* de Bary) y la podredumbre marrón (*Cadophora gregata* (Allington & Chamb.) Gams). En el caso de las raíces, se reporta el síndrome de muerte repentina o súbita (*Fusarium verguliforme* O'Donnell & Aoki; *Fusarium tucumaniae* Aoki, O'Donnell, Yosh. Homma & Lattanzi; *Fusarium brasiliensis* Aoki & O'Donnell y *Fusarium crassitipitatum* Scandiani, Aoki & O'Donnell), el tizón/marchitez/podredumbre de raíz por *Fusarium* spp., la podredumbre carbonosa (*Macrophomina phaseolina* (Tassi) Goid.), el tizón por *Sclerotium* (*Sclerotium rolfsii* Sacc.) y la podredumbre por *Rhizoctonia* (*Rhizoctonia solani* Kühn) (Stewart y Rodríguez, 2013). Entre los patógenos encontrados con mayor frecuencia se encuentran: *P. manshurica* y *P. savastanoi* pv. *glycinea* en hoja; *D. caulivora* y *S. sclerotiorum* en tallo y *M. phaseolina* en raíz (Vidić y Jasnić, 2008). (**Figura 1**)

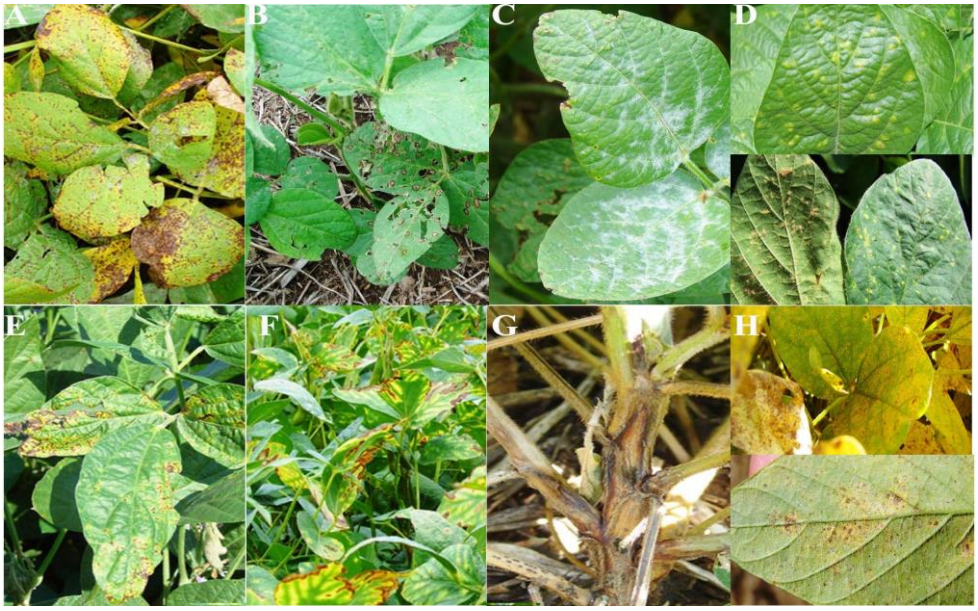

Figura 1. **Síntomas de enfermedades que afectan el cultivo de la soja** A) Septorioris, B) Mancha en ojo de rana, c) Oídio, d) Mildiú, e) Tizón bacteriano, f) Síndrome de muerte repentina o súbita, g) cancro del tallo y h) roya. Tomado de Stewart y Rodríguez, 2013

Por otro lado, se encuentra la incidencia de plagas de orugas cortadoras (*Anticarsia gemmatalis* (Hübner) y *Rachiplusia nu* (Guenée), oruga gata peluda norteamericana (*Spilosoma virginica*), grillo subterráneo (*Anurogryllus muticus*), babosa gris (*Deroceras reticulatum*), babosa negra (*Arion hortensis*), trips (*Caliothrips phaseoli*), bicho bolita *(Armadillidium vulgare)*, barrenador del brote (*Crocidosema aporema*) y chinche verde (*Nezara viridula)* (Aragón, 2003).

Enfermedad del cancro del tallo de la soja

El cancro del tallo de la soja es causado por diferentes especies del complejo *Diaporthe/Phomopsis*: *D. caulivora*, *D. aspalathi* y *D. longicolla* (Pioli *et al*., 2003; Mathew *et al*., 2015; Mena *et al* 2020). El agente causal pertenece a la familia Diaporthaceae, orden Diaporthales, subclase Sordariomycetidae, clase Sordariomycetes del filo Ascomycota del reino fúngico (www.speciesfungorum). Las especies del género *Diaporthe* han sido identificadas en todo el mundo y pueden ser saprófitos, endófitos o patógenos de plantas (Udayanga *et al*., 2012; Santos *et al*., 2017). El complejo *Diaphorte/Phomosis* está conformado por hongos hemibiotrofos con dos fases: la imperfecta, asexual o anamorfo *Phomopsis* y la perfecta, sexual o teleomorfo *Diaporthe* (Pioli *et al*., 2001). El nombre del género *Diaporthe* o *Phomopsis* se ha utilizado por igual y es bien conocido entre los patólogos de plantas. En este sentido, Rossman *et al*. (2015) propuso que el nombre del género *Diaporthe* se conservara sobre *Phomopsis* dado que se introdujo primero.

El cancro del tallo fue detectado por primera vez en los estados del sur de Estados Unidos en 1970 y se identificó como *D. phaseolorum* var. c*aulivora (*Backman *et al.*, 1981; Snow *et al.*, 1984). En 1982 se evaluó la patogenicidad en aislados del sur y del norte de EUA, llegando a distinguir razas fisiológicas y diferencias en preferencias de temperatura entre ellos, que posteriormente dieron lugar a la diferenciación entre *D. phaseolorum* var. *meridionalis* (cancro del sur de EUA) y *D. phaseolorum* var. c*aulivora* (cancro del norte de EUA) (Keeling, 1982; Keeling, 1985; Keeling, 1988; Morgan Jones,

1989), y a la clasificación como especies del hongo; hoy denominados *D. aspalathi* y *D. caulivora*. **(Figura 2).**

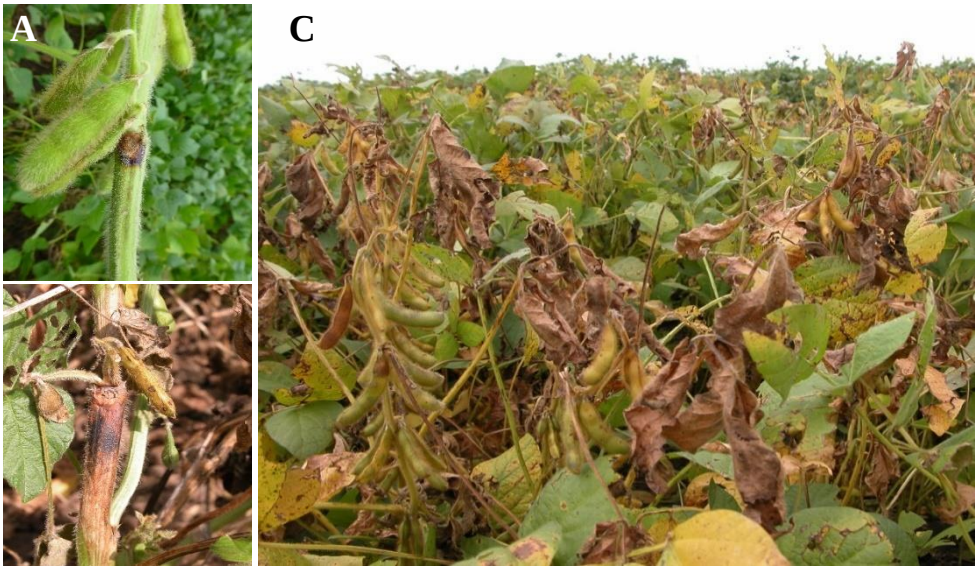

Figura 2. Síntomas de *D. caulivora* en plantas de soja A) Primeros estadíos, B) Lesiones avanzadas en el tallo, C) Severidad de la enfermedad a campo. Fotos de D. Mueller y C. Grau tomado de: https://cropprotectionnetwork.org/resources/articles/diseases/stem-canker-of-soybean

En la región, específicamente en Argentina, se determinó que el cancro del tallo era causado por *D. phaseolorum* var. *meridionalis* (Ivancovich, 1992). En 1999 se identifica por primera vez a *D. phaseolorum* var. c*aulivora* en la provincia de Santa Fe (Pioli *et al.,* 2001) y se convierte en agente predominante del cancro del tallo, debido a la utilización de cultivares con resistencia a *D. phaseolorum* var. *meridionalis* (Pioli *et al*., 2002). En 2005 surge de forma epidémica en Buenos Aires con una prevalencia del 94% e incidencias de hasta 55,5% en el centro y sureste bonaerense (Grijalba *et al.*, 2011; Lago, 2010). En Brasil, el cancro del tallo fue identificado por primera vez en 1989, causado por *D. phaseolorum* var. *meridionalis*, llegando a ocasionar pérdidas de hasta 100% (Yorinori, 1991; Yorinori, 1996). Posteriormente, en 2006 se encontró *D. phaseolorum* var. *caulivora* en Rio Grande do Sul (Costamilan *et al.*, 2008). En Uruguay se aislaron especies de *Diaporthe* de plantas enfermas durante las cosechas 2012-2013 y en el 2015 se realizó el primer reporte de cancro del tallo de la soja con una prevalencia de *D. caulivora* del 83% (Stewart, 2015). En Buenos Aires, Argentina la prevalencia fue del 61% (Sánchez *et al.*, 2016). La enfermedad puede reducir en un 45% el número de granos y un 8% el peso de 1000 granos (Ridao, 2016). En el informe sanitario de la soja en la zona norte de la provincia de Buenos Aires, el cancro del tallo tuvo una prevalencia del 50% entre las enfermedades de la región, sin embargo su incidencia no superó el 8% (Lavilla e Ivancovich, 2018).

El cultivo de soja es suceptible al cancro del tallo en todo su ciclo de desarrollo (Agrios, 2005). Desde el contacto inicial del patógeno hasta la infección existe un período de incubación que oscila entre los 35 y 45 días. La infección puede ocurrir en cualquier estado del desarrollo de la planta debido a que tiene un largo período de incubación (60-70 días) (Ploetz y Shokes, 1985; Lago, 2010). Los síntomas se encuentran localizados en el tallo de las plantas y existen diferencias en la sintomatología dependiendo del agente causal. Los síntomas iniciales del cancro del tallo son pequeñas lesiones marrón

rojizas en el punto de inserción de ramas y pecíolos, visibles a los 15 a 20 días posteriores a la infección. Las lesiones evolucionan lentamente, pasando a una coloración castaño que forma lesiones alargadas, generalmente de un solo lado del tallo que se expanden en ambos sentidos del tallo. Las lesiones llegan hasta la médula produciendo la muerte de la planta. En un corte longitudinal del tallo se observa pudrición y necrosis de los vasos conductores y la médula se torna de color castaño/castaño-rojiza. En las hojas se observa con frecuencia un marchitamiento con clorosis y necrosis internerval (Pioli *et al*., 2002; Stewart y Rodríguez, 2013; Mena *et al*., 2020). En presencia de *D. caulivora* los síntomas de cancros aparecen con bordes difusos, escasos picnidios y peritecios agrupados sobre vesículas estromáticas. Sin embargo, los tallos infectados con *D. aspalathi* presentan síntomas con bordes definidos con picnidios abundantes y peritecios solitarios (Pioli *et al*., 2003).

Las condiciones ambientales favorables para el desarrollo de la enfermedad oscilan entre 20 y 30°C. *D. caulivora* prefiere temperaturas más frescas, entre 20 y 25ºC, mientras que *D. aspalathi* prefiere temperaturas más cálidas, entre 25 y 30ºC (Keeling, 1988). Además, el hongo requiere de precipitaciones o la formación de una película de agua sobre el tejido foliar de 24 a 48 horas para el desarrollo de las esporas (Pioli *et al*., 2003).

Pueden existir dos fuentes de inoculación mediante esporas sexuales (ascosporas, contenidas en las ascas de los peritecios) y asexuales (conidios, contenidas en los picnidios). Sin embargo, en estudios realizados en Argentina ninguno de los aislamientos obtenidos de *D. caulivora* produjo picnidios en su fase asexual (Pioli *et al.,* 2003; Grijalba y Guillin, 2007; Lago, 2010; Benavidez *et al*., 2010), por lo que la enfermedad es considerada del tipo monocíclica, ya que no hay ciclos secundarios de la enfermedad (Pereira y Dhingra, 1997). Sin embargo, como se detallará más adelante en los resultados de esta tesis, se observó la formación de picnidios con liberación de conidios sobre hojas inoculadas en condiciones controladas (Mena *et al*., 2020). La principal fuente de inóculo es mediante ascosporas dispersadas por el viento y salpicaduras de lluvia. El ciclo del patógeno cuenta con cinco etapas: 1. supervivencia y diseminación, 2. contacto y penetración, 3. infección, 4. invasión y colonización y 5. reproducción (**Figura 3)** (Pioli *et al*., 2003).

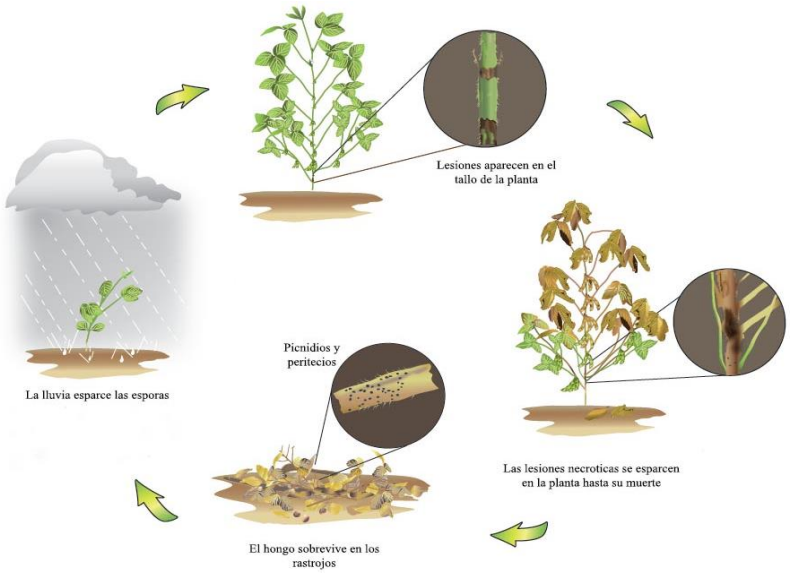

Figura 3. Ciclo del cancro del tallo de la soja, causado por *D. caulivora.* Esquema tomado y modificado de: https://cropprotectionnetwork.org/resources/articles/diseases/stem-canker-of-soybean

Manejo de la enfermedad e identificación de genes de resistencia

Entre las prácticas culturales de manejo que limitan el desarrollo de la enfermedad se encuentran: fechas de siembra temprana para que los estadíos de la planta previos a la floración no coincidan con condiciones climáticas favorables para el patógeno (temperaturas frescas y elevada humedad relativa ambiente); la rotación de cultivos, ya que el monocultivo ha demostrado aumentar la incidencia de la enfermedad; la eliminación de fuentes de inóculos; el control químico sobre fuentes de inóculo (semillas y rastrojos en pre-siembra y post-emergencia); la siembra de cultivares de ciclo corto o tolerantes a la enfermedad (Ivancovich y Botta, 2003). Además, se emplean estrategias basadas en el uso de fungicidas y la resistencia genética (Pioli *et al*., 2003).

Para la identificación de cultivares con resistencia genética al patógeno, se realizan inoculaciones artificiales, se seleccionan cultivares y se evalúan poblaciones segregantes para genes R de resistencia incorporados por cruzamientos. De esta manera se han identificado cuatro genes de resistencia para *D. aspalathi* numerados del 1 al 4 (*Rdc* 1-4). Se determinó que estos genes de soja confieren resistencia a *D. aspalathi* pero no a *D. caulivora*, por lo que fueron redenominados de *Rdc1-4* a *Rdm1-4* (Pioli *et al*., 2003). Posteriormente, Chiesa *et al*. (2009) demostraron la existencia de una región genómica compleja de resistencia raza específica en el cultivar de soja Hutcheson con al menos dos genes: *Rdm4* y un nuevo gen *Rdm5* que confieren resistencia específica a aislamientos de la variedad *D. aspalathi*. Recientemente, se identificó el gen *Rdc1* asociado al cromosoma 13 de *G. max* que confiere resistencia a *D. caulivora* (Peruzzo *et al*., 2019). Sin embargo, no existen genotipos de *G. max* comerciales con resistencia a la enfermedad causada por *D. caulivora*. Por ello, se continúa con la identificación de genes de resistencia y el diseño de estrategias de mejoramiento que partan de la activación de mecanismos de defensa intrínsecos de la planta frente al patógeno.

La especificidad de respuesta de las plantas ante un patógeno, se divide en dos grupos o categorías: no específica o compatible (donde se ubican los fenotipos susceptibles y parcialmente resistentes) y la específica o interacción incompatible (donde están los fenotipos resistentes). En soja se ha reportado el cultivar Williams como susceptible a *D. caulivora* (Thickett *et al.*, 2007; Mena *et al*., 2020), *P. pachyrhizi* (Tremblay *et al*., 2011), *Phytophthora sojae* (Lin *et al*., 2014). Entre los cultivares más resistentes a *D. caulivora* figuran Leo, Coles, AC Hime (Thickett *et al.*, 2007). Siendo Genesis 5601 (antes Leo 1706-07) resistente al patotipo de *P. sojae* más frecuente de Uruguay (Echeverrigaray *et al*., 2017).

Caracterización molecular y genotipado de especies de *Diaporthe*

La determinación y caracterización de hongos del complejo *Phomopsis/ Diaporthe* es difícil debido a su morfología, patogenicidad variable y amplio rango de hospederos (van Niekerk *et al*., 2005). La identificación y caracterización de *Diaporthe* spp. se realizaba principalmente mediante estudios morfológicos, la observación de características de crecimiento, estructuras reproductivas y asociación con el hospedero (Morgan Jones, 1989; Udayanga *et al*., 2012; dos Santos *et al*., 2016). Este enfoque dificultó la discriminación confiable entre miembros del género, dado que muchos de estos hongos son asexuales con baja especificidad de hospedero (Rehner y Uecker, 1994; Murali *et al*., 2006).

El uso de técnicas moleculares ha permitido una mejor identificación de las especies fúngicas. En este sentido, el análisis de regiones nucleares del ADN ribosomal es considerado como un marcador universal para la identificación de especies fúngicas, similar a un "código de barras" (Schoch *et al*., 2012; dos Santos *et al*., 2016). En el género *Diaporthe* se han realizado análisis filogenéticos multi-locus para ver la relación entre especies (Gomes *et al*., 2013; Huang *et al*., 2013; Gao *et al*., 2014; Udayanga *et al*., 2014; Udayanga *et al*., 2016). Las secuencias más frecuentemente utilizadas para estos estudios son: la subunidad mayor del ADN ribosomal (LSU), espaciadores intergénicos del ADN ribosómico (IGS), espaciador transcrito interno del ADN ribosómico (ITS), factor de elongación de la traducción (EF1-α), el gen de β-tubulina, el gen de histona-3 (HIS), el gen de la calmodulina (CAL), el gen de la actina (ACT), el gen de ADN-liasa (APN2), el gen de la proteína ribosomal L37 del 60s (FG1093) y genes del tipo de apareamiento (MAT-1-1-1 y MAT-1-2-1) para mejorar la precisión de la identificación de especies (Zhang *et al*. 1997; van Niekerk *et al*. 2005; Santos *et al*. 2010, Santos *et al* 2017).

En este sentido, Zhang *et al*. (1997) desarrollaron técnicas moleculares tradicionales (PCR-RFLP) y de PCR en tiempo real para caracterizar e identificar aislamientos del complejo *Phomopsis/ Diaporthe* en soja (Zhang *et al*., 1997; Zhang *et al*. 1999). Por otro lado, dos Santos *et al*. (2015) desarrolló y aplicó IRAP (del inglés, Inter-Retrotransposon Amplified Polymorphism) y REMAP (del inglés, Retrotransposon-Microsatellite Amplified Polymorphism) para la evaluación de *Diaporthe* spp. Grijalba *et al*. (2011) amplificaron fragmentos (PCR-RFLP) de la región ITS de ADN ribosomal, seguida por la restricción con una única enzima (AluI) para la identificación de los agentes causales del cancro del tallo de la soja (*D.caulivora* y *D. aspalathi*). La misma técnica fue utilizada posteriormente por Stewart (2015) en la caracterización taxonómica y análisis de la variabilidad de *Diaporthe phaseolorum* en aislados de Uruguay.

Por otro lado, se utiliza el análisis de las regiones con microsatélites o SSR (Simple Sequence Repetitions) o ISSR (Inter-Sequence Single Repetition). Los SSR son repeticiones en tándem de di, tri o tetranucléotidos que se encuentran distribuidos al azar en los genomas de eucariotas. Son muy polimórficos y específicos de cada especie, por lo que constituyen una herramienta útil para el estudio de la diversidad intraespecífica (Reddy *et al*., 2002). Los ISSR consisten en la amplificación de dichas regiones mediante PCR, empleando cebadores cuya secuencia blanco es el microsatélite. Una vez que los productos son analizados mediante electroforesis en gel de agarosa o poliacrilamida, se obtienen patrones de bandas correspondientes a las distintas variantes analizadas (Huang y Sun, 2000; Joshi *et al*., 2000).

La caracterización de aislamientos de *D. caulivora* es necesaria a la hora de evaluar si existen distintas variantes circulando y si las mismas poseen diferente virulencia, aspectos que contribuyen a comprender las características y dinámica de la enfermedad en la región. En este sentido, algunos grupos de investigación secuenciaron los genomas de especies de *Diaporthe* para conocer más de su biología molecular, sin embargo, no se había secuenciado el de *D. caulivora.* Entre los genomas de *Diaporthe* secuenciados se encuentran: *D. longicolla* (Li *et al*., 2015a; Li *et al*., 2015b; Li *et al*., 2017)*, D. aspalathi* (Li *et al*., 2016)*, D. ampelina* (Savitha *et al*., 2016)*, D. helianthi* (Ruocco *et al*., 2018)*, D. capsici* (Fang *et al*., 2020)*, D. phragmitis* (Wang *et al*., 2021), *D. destruens* (Huang *et al*., 2021)*, D. citriasiana, D. citrichinensis* y *D. citri* (Gai *et al*., 2021). El estudio comparativo de los genomas de patógenos acelera la identificación de efectores y factores de virulencia (Klosterman *et al*., 2016), permite analizar cuales son conservados o específicos, conocer su función y agruparlos en familias (Saunders *et al*., 2012; Gebreil

et al., 2016;). La identificación de los mecanismos de patogenicidad permite el desarrollo de estrategias para el mejoramiento genético su aplicación en la agricultura.

Respuesta de defensa a patógenos en plantas

Las plantas exhiben un sistema inmune o de defensa ante la infección por patógenos que involucra el reconocimiento, la activación de señales y la síntesis de proteínas y metabolitos de respuesta que dependen del tipo de microorganismo (Dangl y Jones, 2006). Cuando los patógenos entran en contacto con la planta, la estructura y composición química constitutiva de la planta es la primera línea de defensa. Estas barreras las constituyen estructuras como los apéndices epidérmicos (pelos, escamas, papilas), espesor, dureza y composición química de los tejidos epidérmicos, cantidad y calidad de la cera y cutícula que recubre las células epidérmicas, así como el tamaño y densidad de estomas y lenticelas (Agrios, 2005).
Se han descrito dos tipos de respuesta de defensa: PTI (PAMP Triggered Immunity), donde la planta reconoce patrones moleculares asociados a patógenos (PAMPs; Pathogen-associated molecular patterns) o al daño (DAMPs; Damage-associated molecular patterns), mediante receptores de membrana (PRRs; Pattern recognition receptos) y ETI (Effector Triggered Immunity) en la que se reconocen efectores del patógeno en el interior de la célula vegetal mediante receptores intracelulares (Macho y Zipfel, 2014). Estudios recientes evidencian que la PTI y la ETI pueden actuar de forma conjunta y converger en los mecanismos de respuesta con distinta amplitud y dinámica (Yuan *et al*., 2021). En ambas respuestas de defensa se desencadenan cascadas de señalización de quinasas, flujo de Ca2+, activación de vías de señalización hormonal, acumulación de compuestos antimicrobianos, acumulación de especies reactivas de oxígeno (ROS) y de nitrógeno, reforzamiento de paredes celulares y transcripción de genes específicos de defensa (**Figura 4**) (Ponce de León y Montesano, 2013).

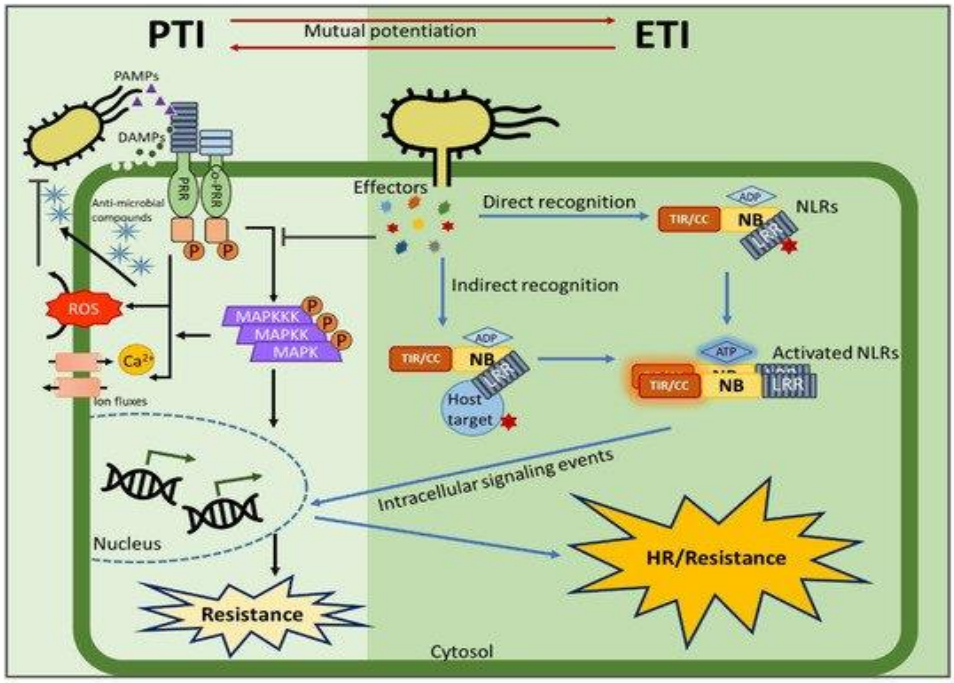

Figura 4. Esquema simplificado de la respuesta de defensa en plantas. PTI (PAMP Triggered Immunity) es activada por patrones moleculares asociados a patógenos (PAMPs; Pathogen-associated molecular patterns) o al daño (DAMPs; Damage-

associated molecular patterns), mediante receptores de membrana (PRRs; Pattern recognition receptos). Efectores de patógenos (estrellitas colores) activan la ETI (Effector Triggered Immunity) al ser reconocidos por receptores ricos en leucina (NLRs). Se activan cascadas de kinasas (MAPKs), flujo de Ca2+, producción de especies reactivas de oxígeno (ROS). PTI y ETI actuan de forma conjunta y potencian la respuesta inmmune. Tomado de (Nguyen *et al*., 2021).

Las hormonas vegetales regulan procesos fisiológicos durante el desarrollo, la reproducción y la respuesta a factores bióticos y abióticos. El ácido salicílico (SA), el ácido jasmónico (JA), y el etileno (ET) están relacionadas con la defensa a patógenos y diferentes tipos de estrés abiótico (Pandey y Senthil-Kumar, 2017). En general, las respuestas mediadas por SA están asociadas con la resistencia a patógenos biotróficos, mientras que JA y ET se consideran señales importantes en la resistencia a patógenos necrotróficos. Otros estudios han demostrado la importancia del ácido abscísico (ABA) y las auxinas en la respueta al ataque de patógenos (Robert-Seilaniantz *et al*., 2011). Las vías de SA y ABA son antagónicas, mientras que ABA y JA pueden ser sinérgicas o antagónicas (**Figura 5**) (Pieterse *et al*., 2012).

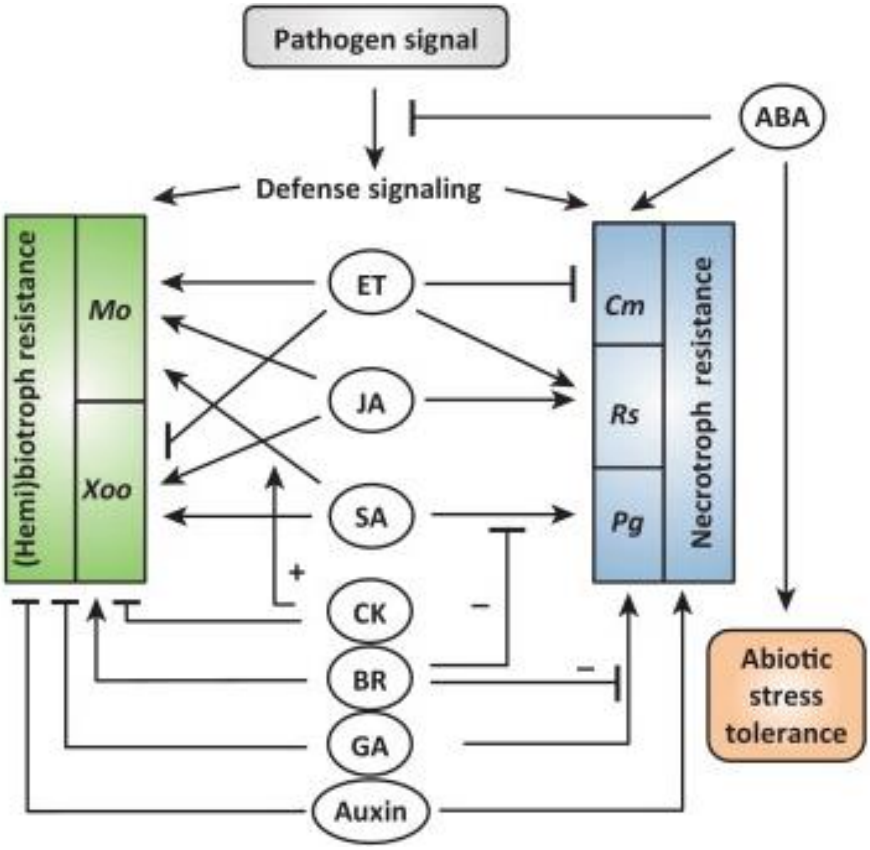

Figura 5 Esquema sobre la relación de hormonas en plantas frente a patógenos. La regulación positiva (flecha) y negativa (barra). La interacción sinérgica (+) y antagónica (-). Abreviaturas de hormonas: ácido abscísico (ABA), brasinoesteroides (BR), citoquinina (CK), etileno (ET), giberelina (GA), ácido salicílico (SA) y ácido jasmónico (JA). Abreviaturas de patógenos: *Cochliobolus miyabeanus* (Cm), *Magnaporthe oryzae* (Mo), *Pythium graminícola* (Pg), *Rhizoctonia solani* (Rs) y *Xanthomonas oryzae* pv. *oryzae* (Xoo). Tomado de (De Vleesschauwer *et al*., 2013).

Varias vías metabólicas son activadas en respuesta a la infección. Una de las más conocidas es la vía de los fenilpropanoides la cual da lugar a la síntesis de varios metabolitos secundarios como fenoles, flavonoides y lignina, los cuales tienen actividades antimicrobianas, limitan la entrada de los patógenos mediante el fortalecimiento de la pared celular y mediante esta vía se podrían generar precursores de la síntesis de SA (**Figura 6**) (Wuyts *et al*., 2006; Piasecka *et al*., 2015).

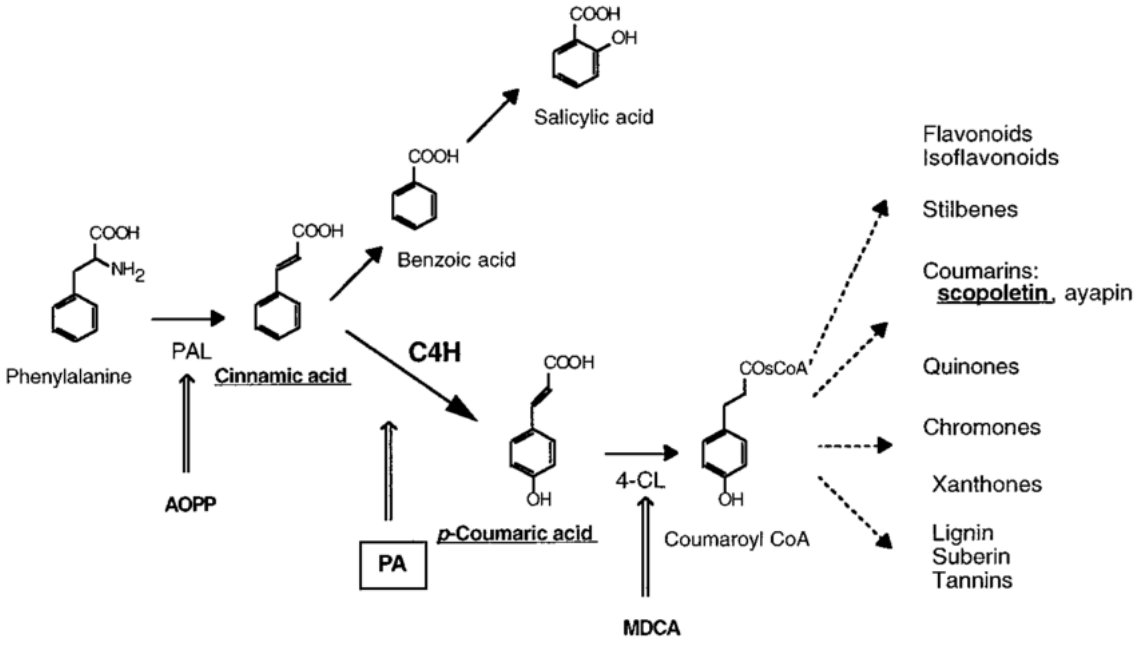

Figura 6. **Esquema general de la vía de los fenilpropanoides.** Se representan las ramas para la producción de ácido salicílico y la síntesis de diversos metabolitos secundarios (flavonoides, isoflavonoides y lignina). Tomado de (Schalk *et al*., 1998)

Por otro lado, den tro de las respuestas de defensa está la activación de genes que codifican para proteínas relacionadas relacionadas con la patogenicidad (PR). Estas se producen durante el desarrollo normal de las plantas o como parte de la defensa inducida por patógenos. Se agrupan en 17 familias dependiendo de su similitud en las secuencias codificantes y/o actividades enzimáticas o biológicas (van Loon *et al*., 2006; Ferreira *et al*., 2007). Hay al menos diez familias de PR cuyos miembros tienen actividades directas contra hongos patógenos, incluyendo PR-1, PR-2 (β-1,3 glucanasas), PR-3, PR-4, PR-8 y PR-11 (quitinasas), PR-5 (taumatina), PR-10 (tipo ribonucleasa) (Chen *et al*., 2010). Además, se encuentran la PR-12 (defensinas), PR- 13 (tioninas) y PR-14, (proteínas de transferencia de lípidos). Las proteínas PR-1 y PR-5 también muestran actividad dirigida específicamente contra oomicetos (van Loon *et al*., 2006).

Análisis transcriptómicos para el estudio global de genes involucrados en la respuesta de defensa frente a patógenos

La tecnología RNA-Seq es una herramienta transcriptómica que permite el secuenciamiento masivo de ADNc o ARN, mediante la cual se obtienen perfiles de expresión génica de las respuestas de defensa. Esta técnica se basa en la secuenciación de alto rendimiento, alta precisión y resultados altamente reproducibles (Wang *et al*., 2009). Además de exactitud y especificidad en la cuantificación de genes expresados diferencialmente, permite identificar nuevas regiones transcritas y eventos de splicing alternativos (Kim *et al.,* 2011). Su análisis ofrece grandes posibilidades para profundizar en los mecanismos que se activan durante la respuesta immune en las plantas (Soto y López, 2012).

En la última década son numerosos los estudios que utilizan esta herramienta para brindar una interpretación articulada de diversos acontecimientos moleculares que integran las resupuestas de expresión génica inducidas por la interacción planta-patógeno. Se han identificado genes vegetales clave en diferentes interacciones planta-patógeno tanto en plantas modelo como *Arabidopsis* (Nobori *et al*., 2018; Zhang *et al*, 2019), *Medicago truncatula* (Gupta *et al*., 2020), *Physcomitrium patens* (Otero-Blanca *et al*., 2021) y en diferentes cultivos como arroz (Kawahara *et al.,* 2012) canola (Lowe *et al*., 2014), cacao (Texeira *et al*., 2014; Naidoo *et al*., 2018).

El acceso a la secuencia del genoma de la soja de Williams 82 (*Rps*1-k) (Schmutz *et al.*, 2010) y los estudios de RNA-seq han permitido la identificación de genes expresados durante la interacción entre soja y diferentes patógenos. Kim *et al.* (2011) analizaron los transcriptomas de dos líneas casi isogénicas (NIL, del inglés *Near Isogenic Lines*) de soja, una resistente y una susceptible a la pústula bacteriana de la hoja causada por *Xanthomonas axonopodis* pv. *glycines*, identificando un total de 2,761 genes expresados diferencialmente; incluyendo un conjunto de genes de respuesta de defensa como las proteínas relacionadas con la patogénesis (PR-1, PR-3, PR-6, PR-12 y PR-14), componentes de señalización de ácido jasmónico MYC2 y los genes jasmonate zim-motif (JAZ). Por otro lado, otro grupo de investigación (Lin *et al*., 2014) caracterizaron genes de vías reguladoras asociadas con la resistencia a *Phytophthora sojae* en diez NILs de soja, y describió las "huellas dactilares" funcionales de las respuestas de resistencia mediadas por *Rps* a través del análisis transcriptómico comparativo. Además, observaron variaciones en los genes vegetales expresados diferencialmente entre las líneas NILs, implicados en la señalización de etileno, ácido jasmónico, ROS y MAPKs con un rol importante en la respuesta de defensa (Lin *et al*., 2014). Con el uso de la transcritómica, se comprobó la asociación de RLP, RLK, NBS-LRR a genes *Rbs* de resistencia y la inducción de factores de transcripción en soja frente al patógeno *Cadophora gregata* (McCabe *et al*., 2018). Mediante RNA-seq fue evidente el incremento en la inducción de la vía de los fenilpropanoides en plantas de soja frente a *Sclerotinia sclerotiorum* (Ranjan *et al*., 2019); la inducción de PRs, endoglucanasas, MAPKKs en respuesta a la inoculación con *Fusarium oxysporum* (Lanubile *et al*., 2015), y la inducción de MAPKKs en respuesta a *P. pachyrhizi* y *Peronospora manshurica* (Bencke-Malato *et al*., 2014; Dong *et al*., 2018).

Según la bibliografía disponible hasta el momento no hay estudios de transcriptoma de soja en plantas inoculadas con *D. caulivora*, y no se han identificado los genes de defensa vegetal que se inducen durante esta interacción. En este sentido, el entendimiento de los mecanismos moleculares involucrados en la interacción entre la soja y el hongo *D. caulivora* es importante en el diseño de estrategias de mejoramiento genético para el control del cancro del tallo en soja.

HIPOTESIS

Las hipótesis del trabajo son varias e incluyen:

La diversidad genética de los aislados de *Diaporthe caulivora* identificados en diferentes zonas del país puede estar o no asociada con la severidad de los síntomas del cancro del tallo.

En las plantas de soja susceptibles se activan mecanismos de defensa ante la infección con Diaporthe caulivora, aunque estos no son suficientes para frenar la infección.

D. caulivora puede secretar proteínas codificadas por genes de patogenicidad, los cuales participan en mecanismos de virulencia, varios de los cuales pueden estar presentes en otros hongos y especies del género *Diaporthe*.

La resistencia o susceptibilidad de diferentes genotipos de soja frente a *Diaporthe caulivora* dependerá de los genes de defensa que se activan antes y durante la colonización del patógeno.

OBJETIVOS

Objetivo general

Caracterizar molecularmente la interacción de soja con el hongo *Diaporthe caulivora*, causante del cancro del tallo

Objetivos específicos

Objetivo específico 1: Analizar la variabilidad genética de *Diaporthe* spp. en aislados de Uruguay.

Objetivo específico 2: Determinar los mecanismos de defensa activados en plantas de soja susceptibles ante la infección con *Diaporthe caulivora.*

Objetivo específico 3: Estudiar los mecanismos de patogenicidad de *D. caulivora* mediante la secuenciación de su genoma y el análisis transcriptómico durante la colonización de plantas de soja.

Objetivo específico 4: Analizar la expresión de genes de defensa vegetal de genotipos de soja susceptibles y resistentes a la infección con *Diaporthe caulivora*.

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RESULTADOS y DISCUSIÓN

Cancro del tallo de la soja causado por *Diaporthe caulivora*; diversidad del patógeno, proceso de colonización y activación de la defensa de la planta

La soja es el principal cultivo de exportación en Uruguay, de gran importancia por el valor nutricional y para la industria de sus granos. Una de las principales enfermedades que afecta el cultivo es el cancro del tallo de la soja (CTS), la cual es causada por varias especies del hongo *Diaporthe*. Estudios previos de Stewart (2015) mostraron una alta incidencia de la especie *Diaporthe caulivora* en lesiones de cancro en tallos de plantas de soja en Uruguay. Dada la importancia de esta enfermedad en la región y en otras partes del mundo, nos propusimos caracterizar la interacción de *D. caulivora-Glycine max*.

En este capítulo generamos conocimientos sobre las especies de *Diaporthe* que se encuentran asociadas a lesiones de cancro en plantas de soja. Se confeccionó una colección de 78 aislados de *Diaporthe* sp., provenientes de diferentes regiones del Uruguay. Entre los diferentes aislados se estableció el grado de relación a partir de un árbol filogenético construido en base a regiones conservadas de ITS y el ETF1α; y se agregaron cepas de referencia para *Diaporthe*. Se encontraron cinco especies del complejo *Diaporthe/Phomopsis.* De ellas, *D. caulivora* y *D. longicolla* fueron las predominantes con 33 y 29 aislados respectivamente. Las otras especies identificadas fueron 12 aislados de *D. miriciae,* 3 aislados que se agrupan con *D. endophytica/D.kongii* y un aislado que se agrupa con *D.serafiniae/D. infecunda.* El alto número de aislados de *D. longicolla* asociado a lesiones del cancro coincide con reportes previos como agente causal de la enfermedad (Gebreil *et al*., 2015; Mathew *et al*., 2015; Ghimire *et al*., 2019).

Además, se analizaron las regiones simples repetidas (ISSR) entre los 33 aislados de *D. caulivora*, distinguiéndose tres grupos diferentes. Además, se estudiaron las estructuras reproductoras de *D. caulivora* y se describió su morfología en comparación con otras reportadas en la bibliografía.

Para conocer las bases de la interacción planta-patógeno, pusimos a punto el método de inoculación en condiciones controladas. El método seleccionado, utilizando plug de micelio sobre herida en el tallo, nos permitió ver el desarrollo de los síntomas de la enfermedad en pocos días, la cual es reproducible en el tiempo. Se confeccionó una escala de evaluación de los síntomas para determinar la severidad de la enfermedad a partir de medidas del largo de las lesiones en el tallo. Ajustados estos parámetros se determinó la virulencia de tres aislados de *D. caulivora*, pertenecientes a diferentes clados y por lo tanto diferentes a nivel genético, se midió el largo de las lesiones, el índice de infección y el área bajo la curva del progreso de la enfermedad. En las plantas de soja genotipo Williams inoculadas con los tres aislados de *D. caulivora* se observó el avance de la enfermedad en el tiempo. Entre los aislamientos D57 y D47 no se encontraron

grandes diferencias en el tamaño de las lesiones y la severidad en los tiempos evaluados. Sin embargo, el aislado D08.4 tuvo una evolución más lenta con lesiones de 60 mm a los 14 días posteriores a la infección (dpi) y las plantas no murieron. Los resultados mostraron que los tres aislados tienen diferentes niveles de agresividad, siendo D57 el más agresivo. Otros autores también han observado diferencias en la virulencia de aislados de *D. caulivora* (Pioli *et al*., 2003; Benavidez *et al*., 2010; Brumer *et al*., 2018).

Se seleccionó el aislado D57 para continuar los estudios dada su agresividad. Además, se puso a punto una metodología para determinar la biomasa del patógeno en muestras del tallo de soja mediante qPCR. En las primeras horas de infección observamos como se incrementó la biomasa hasta alcanzar el 50% del tejido de la muestra a las 96 horas posteriores a la infección (hpi).

Mediante estudios de microscopia en soja pudimos describir como las hifas de *D. caulivora* se asocian a los tricomas de las plantas de soja como punto de adhesión y posible punto de entrada a la planta. Esta asociación es consistente con otros reportes para hongos que señalan a los tricomas como sitio de adhesión y entrada a laos tejidos de la planta (Lazniewska *et al*., 2012). Durante el progreso de la enfermedad en las primeras horas (24-96 hpi) pudimos observar en cortes histológicos como *D. caulivora* coloniza el tallo desde la corteza hasta llegar a los haces vasculares de la planta y posteriormente avanzar en ambas direcciones del tallo produciendo necrosis del tejido. Al invadir los haces vasculares, *D. caulivora* produce necrosis del tejido, generando cambios similares a los producidos por *Cadophora gregata* (Impullitti and Malvick, 2014).

Al analizar las respuestas de las plantas, observamos reforzamiento de la pared celular y acumulación de compuestos fenólicos, los cuales actúan como barrera física para frenar el avance de hongos vasculares (Yadeta and Thomma, 2013) en las plantas inoculadas con el patógeno a diferencia de las plantas sin inocular. Además, demostramos la inducción de un conjunto específico de genes relacionados con la defensa vegetal que codifican para proteínas relacionadas con la patogénesis PR-1, PR-10, beta glucanasa (PR-2), quitinasas (PR-3 y PR-4), dos lipoxigenasas, una peroxidasa, una defensina y dos enzimas de la ruta de los fenilpropanoides (fenilalanina amonio liasa (PAL) y chalcona sintasa (CHS)). La inducción de estos genes también se ha observado en soja inoculados con otros patógenos como *D. aspalathi*, *P. pachyrhizi*, *P. sojae* y *Fusarium solani* (Moy *et al*., 2004; Vega-Sánchez *et al*., 2005; Iqbal *et al*., 2005; Zabala *et al*., 2006; Upchurch and Ramirez, 2010; Schneider *et al*., 2011 Fan *et al*., 2017).

Estos resultados describen por primera vez las especies de *Diaporthe* asociadas a plantas de soja con síntomas de cancro del tallo en el Uruguay. Se describe el proceso de colonización de *D. caulivora* en plantas de soja y el progreso de la enfermedad en condiciones controladas. Conocer la variabilidad del patógeno en el área es de gran importancia para las estrategias de control de la enfermedad. Los estudios realizados en plantas inoculadas en condiciones controladas permiten tener un seguimiento del

avance de la enfermedad y comparar entre genotipos de plantas. Además, el estudio de los mecanismos de defensa activados en plantas de soja susceptibles ante la infección con *D. caulivora* constituye un aporte al conocimiento de la interacción *D. caulivora-G. max*.

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Soybean Stem Canker Caused by *Diaporthe caulivora*; Pathogen Diversity, Colonization Process, and Plant Defense Activation

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Soybean is an important crop in South America, and its production is limited by fungal diseases caused by species from the genus *Diaporthe*, including seed decay, pod and stem blight, and soybean stem canker (SSC). In this study, we focused on *Diaporthe* species isolated from soybean plants with SSC lesions in different parts of Uruguay. *Diaporthe* diversity was determined by sequencing the internal transcribed spacer (ITS) regions of ribosomal RNA and a partial region of the translation elongation factor 1-alpha gene (TEF1*a*). Phylogenetic analysis showed that the isolates belong to five defined groups of *Diaporthe* species, *Diaporthe caulivora* and *Diaporthe longicolla* being the most predominant species present in stem canker lesions. Due to the importance of *D. caulivora* as the causal agent of SSC in the region and other parts of the world, we further characterized the interaction of this pathogen with soybean. Based on genetic diversity of *D. caulivora* isolates evaluated with inter-sequence single repetition (ISSR), three different isolates were selected for pathogenicity assays. Differences in virulence were observed among the selected *D. caulivora* isolates on susceptible soybean plants. Further inspection of the infection and colonization process showed that *D. caulivora* hyphae are associated with trichomes in petioles, leaves, and stems, acting probably as physical adhesion sites of the hyphae. *D. caulivora* colonized the stem rapidly reaching the phloem and the xylem at 72 h post-inoculation (hpi), and after 96 hpi, the stem was heavily colonized. Infected soybean plants induce reinforcement of the cell walls, evidenced by incorporation of phenolic compounds. In addition, several defense genes were induced in *D. caulivora*–inoculated stems, including those encoding a pathogenesis-related protein-1 (PR-1), a PR-10, a *b*-1,3-glucanase, two chitinases, two lipoxygenases, a basic peroxidase, a defensin, a phenylalanine-ammonia lyase, and a chalcone synthase. This study provides new insights into the interaction of soybean with *D. caulivora*, an important pathogen causing SSC, and provides information on the activation of plant defense responses.

Keywords: soybean stem canker, *Diaporthe caulivora*, internal transcribed spacer (ITS) ribosomal RNA (rDNA), translation elongation factor 1-alpha gene (TEF1a), disease symptoms, pathogen colonization, cell wall, defense genes

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INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is one of the most important sources of oil and plant protein (Masuda and Goldsmith, 2009). This legume was originated in China, and later introduced in Europe and America (Qiu and Chang, 2010). At present, the major producers of soybean are United States of America (USA), Brazil, Argentina, China, and India (FAO, 2018). In addition to Brazil and Argentina, in South America, soybean is also cultivated in Uruguay, Paraguay, and Bolivia. In 2017, South America´s soybean production totaled 184.503.944 metric tons (FAO, 2018). More than 135 plant pathogens affect soybean worldwide, of which at least 30 have been reported economically important leading to significant yield losses (Vidić et al., 2013). Breeding programs for disease resistance have been established in several countries since genetic resistance, when available, is the best solution for improving global food security by reducing chemical control. Several species from the fungal genus *Diaporthe* Nitschke (asexual morph *Phomopsis*) (Sacc.) cause important diseases in soybean that affect its production, including seed decay, pod and stem blight, and stem canker (Santos et al., 2011). These diseases are commonly referred to as the *Diaporthe*/*Phomopsis* complex. *Phomopsis longicolla* (*D. longicolla*) is the primary agent of seed decay, while *Diaporthe sojae* (*D. sojae*) is the causal agent of pod and stem blight (Fernández and Hanlin, 1996; Sinclair, 1999). Soybean stem canker (SSC) is mainly caused by two different species, *Diaporthe aspalathi* (*D. aspalathi*) (syn. *Diaporthe phaseolorum* var. *meridionalis*) and *Diaporthe caulivora* (syn. *Diaporthe phaseolorum* var. *caulivora*) (Fernández et al., 1999; Pioli et al., 2003; Santos et al., 2011; Udayanga et al., 2015). SSC is one of the most widespread diseases in soybean growing regions in the world, causing in some cases losses of 100% (Backman et al., 1985). Although different fungi are associated to the abovementioned diseases, all can be isolated from infected seeds or tissues with disease (Kmetz et al., 1978; Holland and Abney, 1988; Sinclair, 1991; Sinclair, 1999). Stem canker caused by *D. caulivora* was first reported in USA in the 1970s (Backman et al., 1985). In 1992, the southern and the northern isolates causing SSC in USA were distinguished based on aggressiveness and preference in temperature, dividing the disease in two: northern stem canker (caused by *D. caulivora*) and southern canker (caused by *D. aspalathi*) (Keeling, 1982; Keeling, 1985; Keeling, 1988). The presence of *D. aspalathi* as the causal agent of SSC was reported in Argentina in 1992 (Pioli et al., 1997). *D. caulivora* was found for the first time in South America in 1999 in Argentina (Pioli et al., 2001), and in 2006, it was identified in diseased plants in Brazil (Costamilan et al., 2008). In 2002, *D. caulivora* was widely disseminated in the main soybean- producing region of Argentina, where it coexists with *D. aspalathi* (Pioli et al., 2002). In Uruguay, different isolates of *D. aspalathi* and *D. caulivora* were identified during 2012–2013 in soybean stems with canker lesions (Stewart, 2015). This was the first report of *D. aspalathi* and *D. caulivora* in Uruguay, and interestingly, 83% of the *Diaporthe* isolates causing stem canker were *D. caulivora*. The high prevalence of *D. caulivora* isolates causing SSC in Uruguay is probably due to the use of soybean genotypes, mostly from Argentinian sources, carrying resistance genes that are not effective to *D. caulivora* (Stewart, 2015). This is consistent with previous results showing that Argentinian soybean genotypes carrying Rdm1, Rdm2, Rdm3, and Rdm4 conferred resistance to *D. aspalathi* but not to *D. caulivora* (Pioli et al., 2003). Disease symptoms caused by *D. caulivora* are associated to withered brown leaves and reddish-brown discoloration and necrosis of the lower half of the stem. Yield losses by *D. caulivora* can be significant, especially when canker lesions develop early, leading to plant wilting and death in the middle of the vegetative stage (Vidić et al., 2013).

Differentiation of taxa within the *Diaporthe/Phomopsis* complex based on morphological characteristics such as pigmentation of the colony, presence or type of pycnidia (asexual state), or presence of perithecia (sexual state) is difficult since they are variable (Zhang et al., 1998; Pioli et al., 2003). The use of molecular analysis such as restriction-site variations in the internal transcribed spacers (ITS) of the nuclear ribosomal RNA genes (rDNA), and/or phylogeny inference based on the nucleotide sequence divergence in the ITS regions of the rDNA together with other genomic regions such as the translation elongation factor 1-alpha (TEF1α) gene, has been suitable to distinguish between closely related *Diaporthe* isolates that cannot be separated using morphological characteristics (Zhang et al., 1997; Santos et al., 2010; Udayanga et al., 2014; Santos et al., 2017). Polymerase chain reaction–restriction fragment length polymorphism (PCR- RFLP) analysis allows distinguishing between *D. longicolla*, *D. sojae*, *D. aspalathi*, and *D. caulivora* (Zhang et al., 1998).

Plants have developed various defense strategies to cope with invading pathogens. As part of the defense responses, pathogen-derived signals are perceived by the plant cells leading to the activation of defense genes *via* different signaling pathways. Genes encoding pathogenesis-related proteins (PR proteins) play important roles in the defense response against pathogens (van Loon et al., 2006). PR proteins accumulate at the pathogen infection sites and contribute to systemic acquired resistance (SAR) (Klessig et al., 2018). These proteins have been divided into 17 classes (PR-1–17) on the basis of their amino acid sequence identity, biological activity, or physicochemical properties (van Loon et al., 2006). Members of the PR protein family have enzymatic activities, including β-1,3-glucanase (PR- 2), chitinase (PR-3, -4, -8, and -11), endoproteinase (PR-7), peroxidase (PR-9), or ribonucleases (PR-10), and have shown to exhibit either antibacterial or antifungal activity (Edreva, 2005; van Loon et al., 2006). In soybean, PR-encoding genes are induced in response to different pathogens (Moy et al., 2004; Zou et al., 2005; Upchurch and Ramirez, 2010; Schneider et al., 2011), indicating the involvement of PR proteins in soybean defense. Understanding how soybean plants defend themselves against *D. caulivora* will help to develop breeding and management strategies for resistance against this pathogen.

The focus of this study was to evaluate the diversity of *Diaporthe* species isolated from symptomatic SSC tissues, with an emphasis on *D. caulivora*, which is the main causal agent of

SSC in Uruguay, and to describe *D. caulivora*–susceptible soybean plants interaction. Only few studies related to genotypic diversity of the pathogen have been performed (Pioli et al., 2003), and no information is available describing the activation of soybean defenses in response to *D. caulivora*. Here, we show that *D. caulivora* isolates with different virulence are present in Uruguay, and we describe macro- and microscopically the infection and colonization process in soybean stems. Finally, we show that plant cell walls are fortified after infection and that expression of several defense related genes are activated. Due to the agronomic importance of soybean, understanding the molecular mechanisms underlying
D. caulivora infection and the activation of plant defenses could be useful for managing SSC in soybean plants.

MATERIALS AND METHODS

Sampling and Isolation of Fungi

Isolates of *Diaporthe* species were recovered from canker lesions in 2012–2013 (53 isolates), and 2015 (25 isolates), taken from different plants from different regions of Uruguay, including farms in the Departments of Colonia, San José, Soriano, Flores, Treinta y Tres, Rivera, Salto, Rocha, Paysandú, Canelones, and Florida (Supplementary Figure 1). Symptomatic soybean stems were collected and disinfected by submerging them in 70% ethanol for 30 s. Small tissue fragments from the border of diseased stems were cut out and placed onto petri dishes containing potato dextrose agar (PDA; Difco, Detroit, USA) acidified with 0.2% lactic acid. Plates were incubated at 20°C for several days. Isolates were purified by single hyphal tip and were stored in PDA slants at 4°C.

DNA Extraction, PCR, and Sequencing

Approximately 100 mg of mycelium was removed from 8-day- old cultures grown on PDA medium and ground with liquid nitrogen. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The ITS region of nuclear rDNA and a partial sequence of the TEF1α gene of each isolate was amplified using the universal fungal primers ITS4 and ITS5 (White et al., 1990), and EF1-728F and EF1-986R (Carbone and Kohn, 1999), respectively. The following PCR mix was used: 1X *Taq* buffer [20 mM (NH4)2SO4; 75 mM Tris-HCl pH = 8.8 and 0.01% (v/v)
Tween 20]; 2.0 mM MgCl2; 0.2 mM of each dNTP; 1.0 μM of each primer; 2.5 U of *Taq* polymerase (Thermo Scientific); 50 ng of genomic DNA; and double-distilled water up to 50 μl. A negative control using water instead of DNA was also included. PCR cycling program for both genes consisted of an initial denaturing step for 3 min at 96°C, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and a final extension of 4 min at 72°C. The sizes of the amplified DNA products were determined by electrophoresis in a 1.5% agarose gel in Tris/acetate/EDTA (TAE) at 120 V for 30 min, stained with ethidium bromide, and visualized by UV light. PCR
products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced in both directions at Macrogen (Korea). Obtained sequences were aligned using MEGA7 (Kumar et al., 2016), and initial species identification was done by performing Blast searches of the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov). A summary of the *Diaporthe* species including information related to the name of the isolates (ID), the soybean cultivar, location, year of collection, percentage of similarity, and GenBank sequence accession numbers with the highest similarity is available in Supplementary Table 1. For phylogenetic analysis additional reference sequences for the different *Diaporthe* species identified were retrieved from GenBank (Supplementary Table 2), and two *Diaporthe vaccinii* strains were used as outgroups. In addition, sequences of *Diaporthe* isolates closely related to the identified species were added to the analysis, including *D. aspalathi*, *D. sojae*, *D. helianthi*, and *D. infecunda* (Udayanga et al., 2014; Thompson et al., 2015; Santos et al., 2017).
Phylogenetic relationships between sequences of concatenated and individual gene-trees were performed using Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The optimal model that best fit the aligned sequences data sets was determined using the JModelTest 2 program (Posada, 2008), according to Akaike Information Criterion (AICc). Bootstrap support values based on 1,000 replications were calculated for tree branches construction using the program MEGA7 (Kumar et al., 2016). Sequences were deposited in the GenBank database (MK483139– MK483213, MK507892, and MN584748–MN584826), and the
corresponding access numbers are listed in Supplementary Table 1.

Inter-Sequence Single Repetition–PCR Amplification

DNA extracted from 14 *D. caulivora* isolates obtained in 2015 was amplified using different inter-sequence single repetition (ISSR) primers (Supplementary Table 3) (Weising et al., 1989; Zhou et al., 2001; Ureña-Padilla et al., 2002; Talhinhas et al., 2005; Fan et al., 2010; Barquet et al., 2012). DNA amplification conditions for ISSR-PCR assays included an initial step of 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at primer-specific temperature for 30 s, and elongation at 72°C for 90 s. A final extension was performed at 72°C for 5 min. Each reaction contained: 1X *Taq* buffer [20 mM (NH4)2 SO4; 75 mM Tris-HCl pH 8.8 and 0.01% (v/v) Tween 20]; 2.0 mM of MgCl2, 1 μM of ISSR primer, 0.2 mM of each dNTP, 1.0 U of *Taq* polymerase (Thermo Scientific), 50 ng of genomic DNA, and double-distilled water up to 20 μl. A negative control using water instead of DNA was also included. All ISSR-PCR assays were performed twice. Amplified products were analyzed by electrophoresis on 2.0% (w/v) agarose gels in Tris/borate/ EDTA (TBE) at 90 V for 50 min, stained with GelRed (Biotum, USA), and visualized by UV light (Macro VueUvis-20, Hoefer Inc, USA). Gels were scanned using a Fuji Film Starion FLA 9000 image scanner. Product sizes were estimated based on 100 bp DNA Ladder Plus (BioLabs). The obtained images were analyzed by the GelCompar II Software (Applied Maths, Brazil), where

presence (1) or absence (0) of bands for each oligonucleotide primer combination used in the amplification was scored for the different isolates. Band profile reproducibility was tested by repeating the PCRs three times for the selected isolates and primers tested. A dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) method (Michener and Sokal, 1957). Significance of each cluster was calculated with the cophenetic correlation (Sokal and Rohlf, 1962), which measures the correlation between the similarity measure derived from the dendrogram and the similarity matrix. The internal significance of each cluster was evaluated using the Jaccard method (Jaccard, 1901). The value used for optimization and tolerance of results was 1%.

Morphology and Growth Examination

Morphology and growth of three selected strains were tested in five types of microbiological media, including PDA; SDA (Sabouraud dextrose agar; Britania); YPD (yeast extract–peptone–dextrose; 10 g of yeast extract, 20 g of peptone, 20 g of glucose, and 20 g of agar per liter, pH = 6.6); CMA (Corn Meal Agar; 2 g of corn meal, 15 g of agar and 1% Tween 80 per liter, pH = 6.0); and Czapeck (2 g of sodium nitrate, 0.5 g of potassium chloride, 0.5 g of magnesium sulfate, 0.01 g of iron sulfate, 1 g of phosphate bipotassium, 30 g of sucrose, and 15 g of agar per liter, pH = 6.8). Agar plugs (5 mm in diameter) from the growing edge of 8-day-old cultures grown on PDA were transferred to newly prepared media, and cultures were incubated at 24°C in 16 h light/8 h darkness photoperiod. Three replicates were established for each culture medium. Colony morphology and growth were observed and measured for 6 consecutive days.

A modified protocol was used for perithecial production (Cavinder et al., 2012). Briefly, when mycelium reached the border of the plate, the aerial mycelia was removed with a sterile toothpick, and 1 ml of Tween 60 (2.5%) was added to the surface. This procedure was repeated each time mycelium growth was evident. Development of perithecia was observed weekly, and after approximately 4 weeks perithecia were collected by scraping the surface with Tween 80 (0.1%). Asci and mature ascospores were obtained by macerating the perithecia in an electric blender (Kinematica GmbH Littau- Luzern, Switzerland) with 5 ml sterile 0.1% agar in distilled water. For nuclei staining (Hoechst 33342), asci and ascospores were hydrolyzed in 4 M HCl for 10 min according to Min et al. (2010). Confocal images were recorded on a LSM 800 laser scanning confocal microscope (Zeiss) equipped with a Zeiss Axiocam 506 color digital camera. Excitation/detection was at 405/488 nm. Length of 20 perithecium, 10 asci, and 20 ascospores were analyzed, and measurements were repeated twice from two different plates.

Plant Material and Pathogen Inoculation

The SSC-susceptible soybean (*G. max*) cultivar Williams (PI 548631) was used for all plant assays. This cultivar is also susceptible to other soybean pathogens such as *Phakopsora pachyrhizi* (*P. pachyrhizi*) and *Phytophthora sojae* (*P. sojae*), and analysis of defense gene expression have been performed in response to biotic stress signals and *P. pachyrhizi* (Dorrance et al., 2004; Delgado-Cerrone et al., 2018; https://genevestigator.com/gv/). Three seeds were planted in a 10-cm-diameter pot filled with a mix of soil and vermiculite at a rate of 3:1. Soybean seedlings were grown in a growth room under a 16 h light/8 h dark lighting regime at 24°C. For all experiments, 3-week-old plants at V2 were used. Pathogen inoculation was performed using different methods, including the toothpick method, mycelium and ascospore inoculation, stem wounding, and detached leaf inoculation (Keeling, 1982; Ploetz and Shokes, 1985; Pioli et al., 1997; Costamilan et al., 2008; Benavidez et al., 2010; Sun et al., 2012; Campbell et al., 2017). For the toothpick inoculation method, sterile toothpicks were placed on fresh PDA plates with a PDA plug containing mycelium, and after 10 days, the toothpicks overgrown with mycelium were inserted directly into the stem under cotyledons (approx. 10 mm). A sterile toothpick was inserted into the stem as a control. For the inoculation method involving spraying of mycelium, 10 PDA plugs containing mycelium were placed in 100 ml potato dextrose broth which was agitated during 7 days in dark at 120 rpm. Mycelium was placed in approximately 30 ml of sterile distilled water and macerated in an electric blender. The resulting suspension was filtered through a 40-μm-pore-size sterile cell strainer (Falcon) to remove mycelium lumps and adjusted to optical density (DO) of 1. Soybean plants were sprayed with the corresponding suspension. For the ascospore inoculation method, perithecia were collected as mentioned previously and macerated in an electric blender with 30 ml of sterile distilled water. The resulting suspension was also filtered through a 40-μm-pore-size sterile cell strainer, and spore count was adjusted to 2×10^5 per ml using a hemocytometer (Neubauer Improved Brightline, Germany). A 10 ml drop was placed onto unifoliate leaf petioles. Plants inoculated with the last three methods were placed immediately in a humid chamber for 48 h after inoculation. The stem wounding method was performed by making a thin slice along the stem with a sterile scalpel (approx. 7 mm), 1 cm above the cotyledon, and single agar plugs bearing mycelium were carefully placed on the wound. PDA plugs without pathogen were placed on wounds as a control. In both cases, the wound was sealed with solid petrolatum (Vaseline). For the detached leaf inoculation, petioles with their corresponding trifoliated leaves were placed in Eppendorf vials containing half strength Murashige and Skoog medium inside petri dishes containing wet paper to avoid desiccation. Agar plugs with or without mycelium (control) were placed on midrib leaf-veins (one plug on each trifoliate leaflet), and petri dishes were sealed with parafilm. For all experiments, PDA plugs (5 mm) containing mycelium were taken from the growing edge of 5 days fresh grown cultures.

Isolate Pathogenicity and Development of Stem Canker Symptoms

Based on molecular markers, growth, and morphology, three different isolates of *D. caulivora* (D47, D57, D08.4) were selected for pathogenicity test in plants. These tests were performed by inoculating plants using the stem wounding method as described

above. Development of characteristic SSC symptoms was analyzed until death of the plant. Lesion length (mm) was determined at various time points [3, 5, 7, 11, and 14 days post-inoculation (dpi)], and observations were made to describe disease progress. Ten plants were used per treatment, and the experiment was repeated three times. Disease severity was rated in each individual stem, based on a new proposed scale. The scale includes severity values (Ni) that ranged from 1 to 7 ranked as follow: (1) plants without external symptoms; (2) stem lesions equal to or less than 15 mm; (3) stem lesions equal to or less than 25 mm; (4) stem lesions up to 50 mm in length, sometimes showing foliar symptoms; (5) stem lesions up to 75 mm long, stem girdling, typical interveinal foliar chlorosis; (6) stem lesions larger than 75 mm, plant showing interveinal foliar necrosis; and
(7) dead plants (Supplementary Figure 2). Disease severity index was calculated using the formula: S = $\sum$ (ni/nt × Ni), where S = severity index; ni = number of individual plants rated for severity value Ni; and nt = total number of plants per treatment (Freitas et al., 2002). The area under disease progress curve (AUDPC) was calculated according to Shaner and Finney (1977) at 14 dpi, using the formula: AUDPC = $\sum_{i=1}^{n} = [Y_i + Y_{i+1}) = 2 * (X_{i+1} - X_i)]$ where Yi = severity index according to infection index, Xi = times in days, and n = total observations number; where the final value of AUDPC is the sum of the areas by lapses, which result from the multiplication of the average reading of two consecutive dates (y value) by the lapse (days, x value) between readings. Infection index was calculated by the formula reported by Groth et al. (1999): infection index (%) = 100 *[$\sum$ nb/(N−1)T], where n = number of individual plants in each scale value; b = scale value for each individual plant; N = 7, maximum scale rating; and T = total number of plants evaluated in each treatment. Significant differences between treatments were determined by non-parametric Kruskal–Wallis and Mann–Whitney tests using SPSS Statistics v. 21.0. The significance level for data used was $p < 0.01$.

Visualization of Fungal Colonization and Plant Cell Wall–Associated Responses

Colonization of soybean plants by *D. caulivora* was followed by microscopic observations of stems. Control and inoculated samples were taken approximately 0.5 cm above the wound border at different times after inoculation [8, 24, 48, 72, and 96 h post-inoculation (hpi)]. Tissues were placed in a clearance solution [0.15% trichloroacetic acid (w/v) in ethanol: chloroform (4:1; v/v)] for 48 h, and the solution was changed once during this time. The samples were then washed two times for 15 min with 50% ethanol, two times for 15 min with 50mM NaOH, three times for 10 min with MilliQ water, and finally they were incubated for 30 min in 0.1 M Tris/HCl (pH = 8.5). Samples were stored in 50% (v/v) glycerol until they were cut and stained (Knight and Sutherland, 2011). Stem samples were embedded in 6% agar, and 100 μm transverse and longitudinal sections were obtained with a vibratome. Sections were maintained in 50% (v/v) glycerol until staining. Hyphae of *D. caulivora* were stained with the chitin-specific dye Wheat Germ Agglutinin–Alexa Fluor 488 conjugate (WGA-AF488, Molecular Probes, Invitrogen), and plant cell membranes and cell walls were visualized using propidium iodide (PI; Sigma) (Sun et al., 2014; Redkar et al., 2018). Samples were incubated in staining solution (20 μg/ml PI, 10 μg/ml WGA-AF488, 0.02% Tween 20 in phosphate-buffered saline, PBS) for 30 min and washed in PBS (pH = 7.4). Confocal images were recorded on an FV1000 laser scanning confocal microscope (Zeiss). Excitation/detection was at 488/500–540 nm for WGA-AF488 and at 560/600–700 nm for PI. Bright field microscopy and fluorescence microscopy were performed with an Olympus BX61 microscope (Shinjuku-ku, Tokyo, Japan), and images shown in this study were captured with MICROSUITE software package (Olympus, Tokyo, Japan) (2018). Cell wall modifications were detected with solophenyl flavine 7GFE 500 by staining the stem sections with 0.1% solophenyl flavine 7GFE 500 in water for 10 min, then rinsed in water and visualized with epifluorescence (Oliver et al., 2009). The incorporation of phenolic compounds into the plant cell walls was visualized by staining with safranin-O according to Lucena et al. (2003), and Toluidine blue according to Mellersh et al. (2002).

Quantitative PCR

The fungal biomass in soybean stem of *D. caulivora*-infected plants at 0, 8, 24, 48, 72, and 96 hpi was quantified. Three plants per treatment were used as biological replicates. Samples were frozen in liquid nitrogen, and DNA was extracted from stem tissues (stem section of 1.5 cm including the wounded area) using the DNeasy kit (Qiagen, Hilden, Germany). DNA concentration and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Quantitative PCR (qPCR) was performed using primers designed for the elongation factor gene of soybean (Ef1α) and the β-tubulin gene of *D. caulivora* (Supplementary Table 3) (Miller and Huhndorf, 2005; Upchurch and Ramírez, 2010). Specificity of PCR using these species-specific primer pairs was first confirmed. qPCR was performed using the QuantiNova Probe SYBR Green PCR Kit (Qiagen, Germany) in a 96-well thermocycler (New Applied Biosystems QuantStudio 3). Each reaction consisted in 20 μl containing 10 μl of SYBR Green PCR Master mix (2X), 0.7 μM primers mix, and DNA (~25 ng). The thermocycler was programmed to run for 2 min at 95°C, followed by 40 cycles of 15 s at 94°C and 20 s at 60°C. Water was used as negative control. As a standard, a serial dilution of genomic DNA from *D. caulivora* with known concentrations (60 ng, 6 ng, 600 pg, 60 pg, and 6 pg) were analyzed to determine the sensitivity and linear range of the assay. Pathogen standard curve was generated by plotting the CT values of a 10-fold dilution series of *D. caulivora* DNA stock solution versus the logarithm of the concentration. The resulting regression equations were used to calculate fungal DNA in stem samples. Similarly, a standard curve was generated to estimate the amount of soybean DNA present in each sample. Pathogen β-tubulin estimated was expressed relative to soybean elongation factor. Each data point is the mean value of three biological replicates. Two technical replicates were used for each sample. Student's t-test was applied to all qPCR data, and values of $p \leq 0.01$ were considered statistically significant.

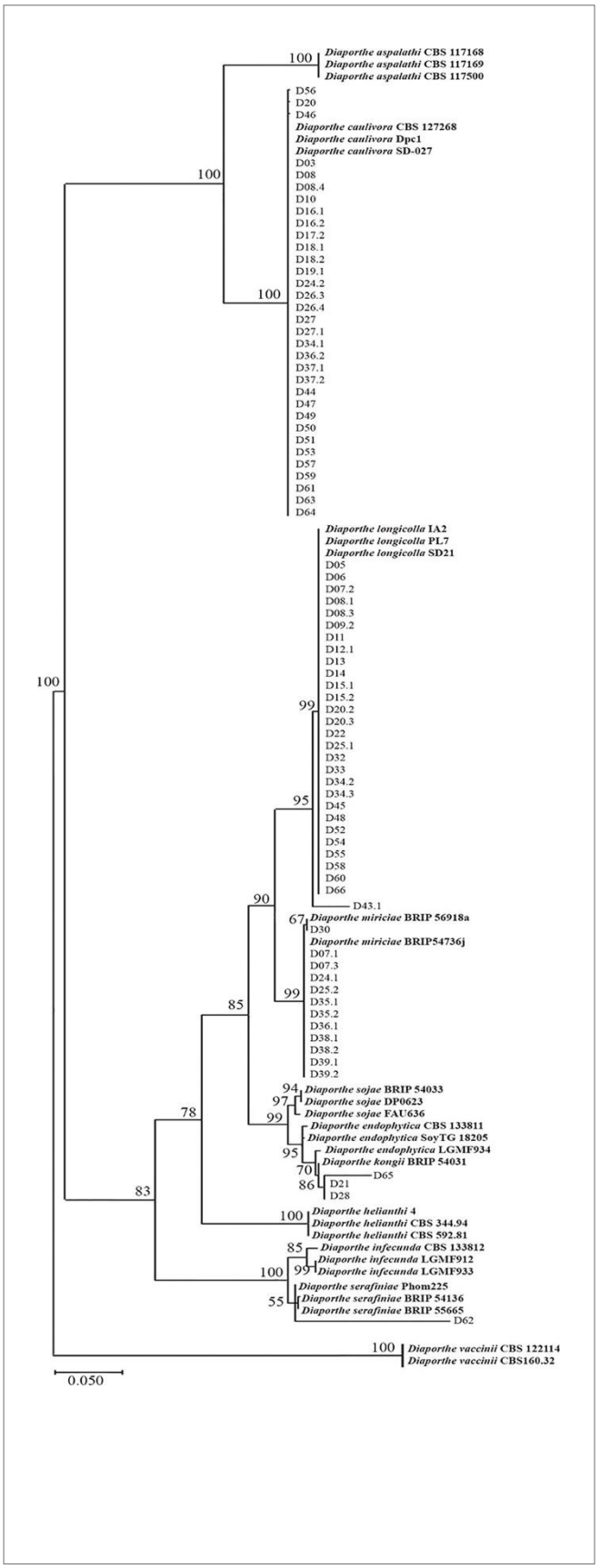
Soybean Defense Gene Expression Analysis and Identification of Cis- Regulatory Elements

Several soybean genes were selected for expression analysis, including *PR-1* (pathogenesis-related protein-1), *PR-2* (*b*-1,3-glucanases), *PR-3* and *PR-4* (chitinases), *PR-10* (Ribonuclease-like protein), *LOX2* and *LOX7* (lipoxygenases), *PDF1.2* (Defensin 1.2), *IPER* (basic peroxidase), *PAL* (Phenylalanine-ammonia lyase), and *CHS* (chalcone synthase). Soybean stems inoculated with *D. caulivora* and control samples (stem section of 1.5 cm including the wounded area), were frozen in liquid nitrogen at 4, 8, 24, and 48 hpi. Samples without any treatment were also taken. Tissues were grounded with liquid nitrogen, and total RNA was extracted from 100 mg of tissues, using the RNeasy Plant Mini according to manufacturer's instructions (Qiagen, Germany). Quality of the isolated RNA was checked by running samples on 1.2% formaldehyde agarose gel. RNA concentration was measured using a NanoDrop 2000c (Thermo Scientific, Wilmington, USA). For cDNA synthesis, 2 *m*g of total RNA were treated with DNase I (Thermo Scientific), and cDNA was synthesized using RevertAid Reverse transcriptase (Thermo Scientific) and oligo (dT) according to the manufacturer's protocol. RT-qPCR was performed in a 96-well thermocycler (New Applied Biosystems QuantStudio 3) using the QuantiNova Probe SYBR Green PCR Kit (Qiagen, Germany). Each 20 µl reaction contained 10 µl of SYBR Green PCR Master mix (2X), 0.7 *m*M primers mix, and 1 µl of template cDNA. The thermocycler was programmed to run for 2 min at 95°C, followed by 40 cycles of 15 s at 94°C and 20 s at 60°C. Water was used as negative control. Ef1*a* was used as the internal control, and expression in control-treated stem tissues was used as the calibrator, with the expression level set to 1. Amplification efficiencies for different primer combinations were analyzed and all were greater than 95%. Relative expression was determined using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Each data point is the mean value of three biological replicates. Two technical replicates were used for each sample. Student's t-test was applied to all RT-qPCR data, and values of $p \leq 0.05$ were considered statistically significant. Primers used for qPCR analyses are provided in Supplementary Table 3. Cis-regulatory elements/motifs were analyzed 1,000 bp upstream from the transcription start site for all soybean defense genes analyzed in this study by using Phytozome v12.1 (http://www.phytozome.net/), and Plant Cis-Acting Regulatory Elements (PlantCARE) databases, http://bioinformatics.psb.ugent.be/webtools/plantcare/html (Lescot et al., 2002).

RESULTS

Diaporthe Isolates Obtained from SSC Lesions

Phylogenetic analysis showed that the 78 *Diaporthe* isolates recovered from SSC lesions in Uruguayan farms belong to five clades supported by high bootstrap values (Figure 1). The first clade consists of 33 Uruguayan isolates and the reference strains of *D. caulivora*. The second clade includes 29 Uruguayan isolates



and the reference strains of *D. longicolla*. The third clade grouped 12 isolates with *D. miriciae* reference strains. The fourth clade has three isolates that grouped with strains of *D. endophytica* and *D kongii*, and the fifth clade includes one isolate that grouped with *D. serafiniae* and *D. infecunda*. The presence of a high number of *D. caulivora* isolates was consistent with previous results showing that *D. caulivora* is the main causal agent of SSC in Uruguay (Stewart, 2015). Conversely, such high number of isolates belonging to *D. longicolla* was not expected. We therefore confirmed the capability of *D. longicolla* to produce stem lesions by inoculation assays (Supplementary Figure 3). None of the isolates corresponded to *D. aspalathi*. Taken together, the results show that *Diaporthe* species associated to stem canker lesions in Uruguay are mainly *D. caulivora* and *D. longicolla*.

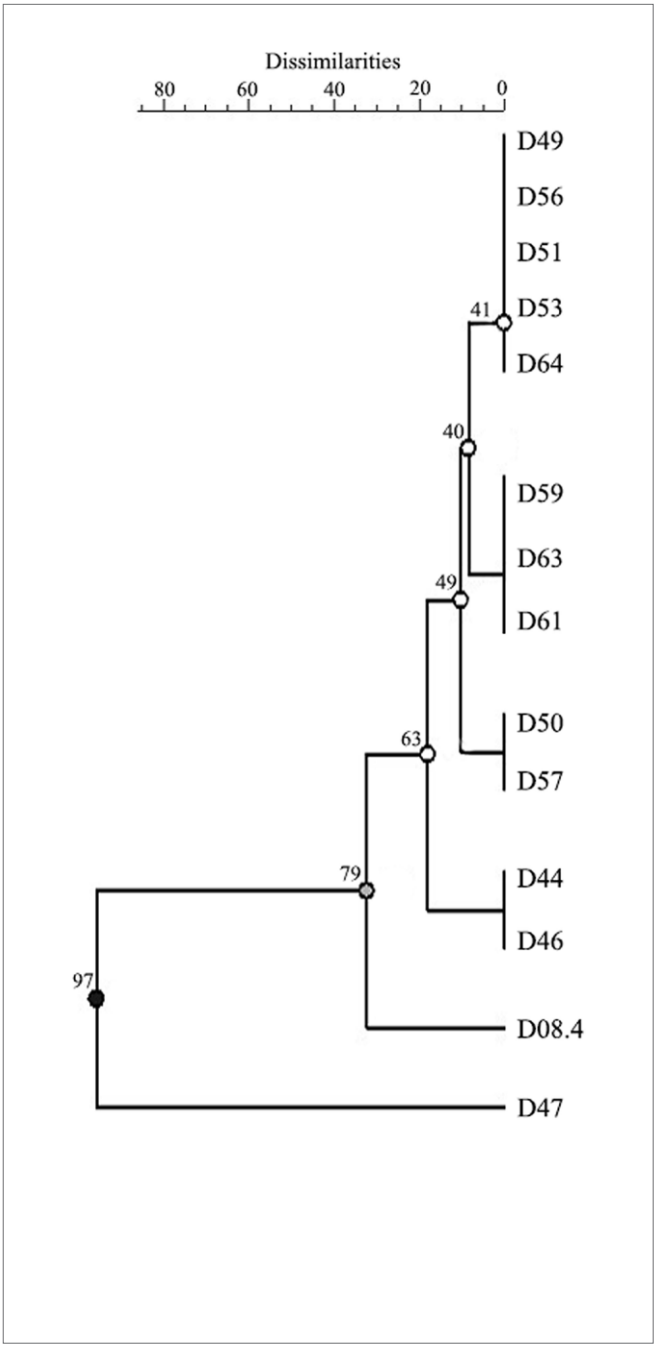
Genetic Diversity of *D. caulivora* Based on ISSR Markers

Since few studies are available on *D. caulivora* and that it is being the most predominant species associated to SSC lesions in Uruguay, we analyzed the genetic diversity using ISSR. Primers (ACTG)4, (GTG)5, and (CAG)5 were chosen due to their informative amplification patterns, and result reproducibility (Supplementary Figure 4). Sixteen informative amplification bands were obtained with these ISSR primers. Although variability among *D. caulivora* was not high, differences between isolates could be distinguished in the dendrogram based on amplification patterns (Figure 2). Isolates were grouped in three clusters, two represented by isolate D47 and isolate D08.4, respectively, and a third cluster containing the remaining isolates. Based on these results, three *D. caulivora* isolates (D47, D57, and D08.4) were selected for further analysis.

Morphology and Growth Characteristics of Selected *D. caulivora* Isolates

Growth characteristics of the three selected *D. caulivora* isolates were analyzed. After growing them on five different culture media, a consistent radiate growth pattern was observed, and no asexual morph was evident (Supplementary Figure 5). No differences in colony morphology between isolates D47, D57, and D08.4 could be detected. *D. caulivora* isolates cultured during 7 days on PDA, SDA, and YPD showed white aerial mycelia withzones ofwhite-brownish pigmentation. On the reverse side of growth plates, the colonies developed a yellow pigmentation with cream to pale brown in the center and a striate growth, especially visible on YPD. On Czapeck medium, mycelia grew translucent, rhizoid-like, without pigmentation or stomata, while mycelia cultured on CMA grew almost transparent, with zonation but without pigmentation, and growth was almost entirely inside the agar (Supplementary Figure 5). Colony growth was fast in PDA, SDA, and YPD, and the three *D. caulivora* isolates reached the border of the plate after 5 days (Supplementary Figure 6). Mycelium growth in CMA and Czapeck was slower, and only isolate D08.4 reached the border at 6 days.

Perithecia on soybean stems were black, globose, smooth, clustered in groups, and immersed in the plant tissue (Figures 3A, B). Similarly, the three isolates were homothallic



and showed clustered perithecia in PDA (Figures 3C–N). Perithecial necks were long and thin in D57 and D08.4, and short and broad in the isolate D47. Necks in D47 were 362.8 ± 27.4 μm long, 97.3 ± 10.5 μm wide at the base, and 45.5 ± 3.0 μm wide at the apex; D57 and D08.4 were 527.6 ± 32.8 and 611.1 ± 34.8 μm long, 75.1 ± 4.8 and 76.2 ± 6.3 μm wide at the base and 46.2 ± 3.7 and 46.6 ± 3.6 μm wide at the apex, respectively (Figures 3I–N). Asci were similar for the three *D. caulivora* isolates; unitunicate, eight-spored, oval to clavate, measuring 28.4 ± 0.7 × 5.8 ± 0.3 μm for D47; 28.8 ± 0.5 × 5.9 ± 0.3 μm for D57; and 28.8 ± 0.3 × 5.8 ± 0.2 μm for D08.4. Ascospores

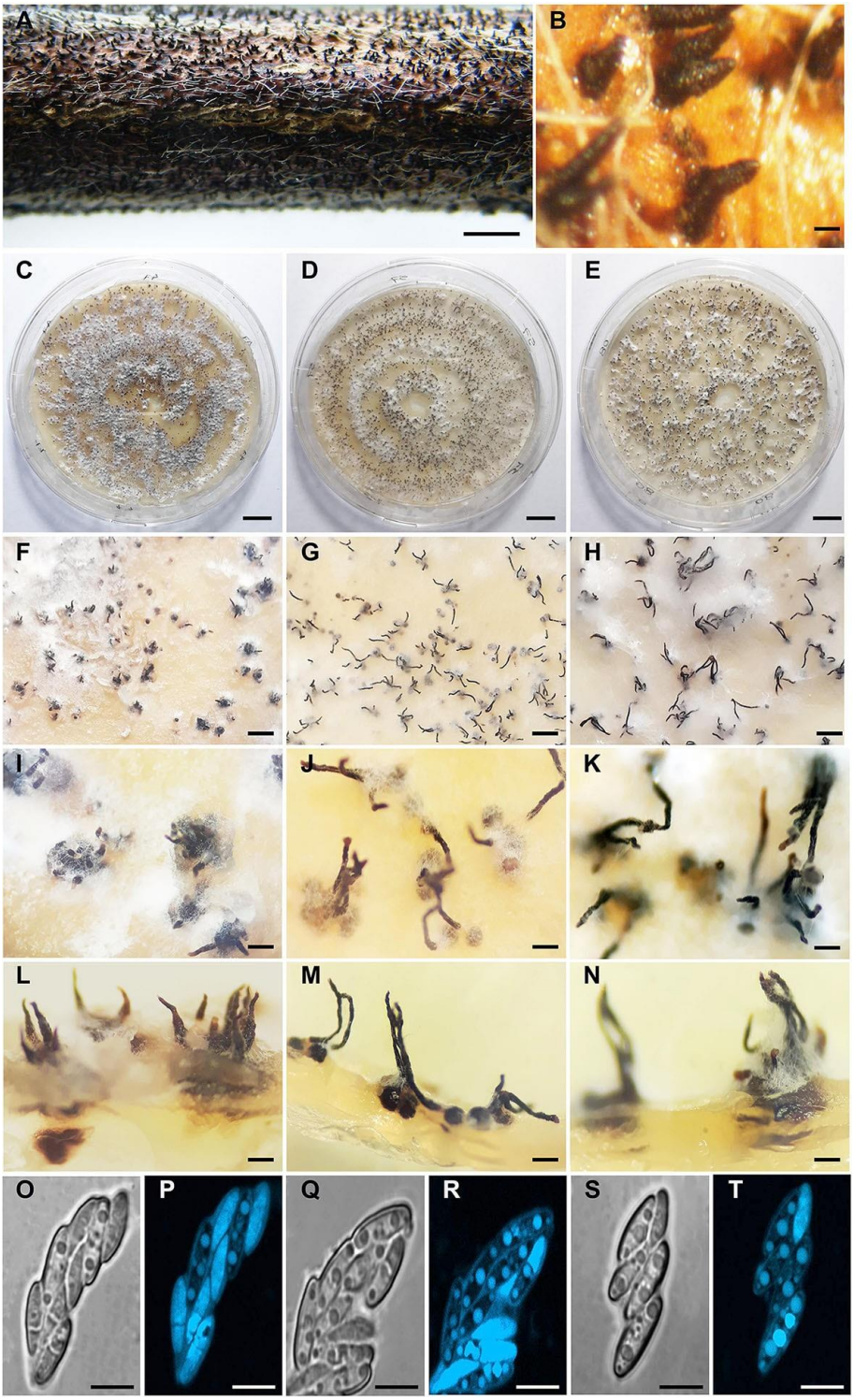


FIGURE 3 | Morphology of *Diaporthe caulivora*. (A) Perithecia of *D. caulivora* (isolate D57) on soybean stem in water agar. (B) Perithecia necks of D57 protruding from soybean stem. (C–N) Perithecia of *D. caulivora* on potato dextrose agar (PDA) medium. (O–T) Asci and ascospores. Isolate D47 (C, F, I, L, O, P), isolate D57 (D, G, J, M, Q, R), and isolate D08.4 (E, H, K, N, S, T). Scale bars: A = 4 mm; B = 400 *m*m; C–E = 10 mm; F-H = 500 *m*m; I–K = 300 *m*m; L–N = 200 *m*m; O–T = 5 *m*m.

were two-celled, hyaline, smooth, ellipsoid to fusoid, medianly septated, slightly to non-constricted, often biguttulate (Figures 3O–T). Ascospore size were similar in the three *D. caulivora* isolates; 8.7 ± 0.2 × 2.1 ± 0.3 μm (D47); 8.6 ± 0.6 × 2.4 ± 0.2 μm (D57), and 9.1 ± 0.7 × 2.2 ± 0.2 μm (D08.4). Taken together, *D. caulivora* isolates were similar, but differ in having distinctive growth characteristics for D08.4 and shorter and broader perithecial necks in D47.

Development of Stem Canker Symptoms

Out of the different methods used to inoculate D57, stem wounding and the application of an agar plug containing mycelium led to the development of SSC with typical brown stem lesions leading to plant decay at 14 dpi (Supplementary Figure 7). While with the toothpick inoculation method plants developed stem lesions, disease did not progress after 14 dpi. With the mycelium and ascospores suspension methods, only some browning of the stem was visible, and typical lesions did not form. Based on these results, the stem wounding method was selected for the rest of the work, and a more detailed analysis of disease progression was performed (Figure 4). Disease development was evidenced by brown discoloration of the stem and withered leaves above de canker lesion (Figures 4E–H). First symptoms of stem canker were observed at 3 dpi, which were more evident at 5 dpi showing typical brown lesions up to 2 mm in length (Figures 4E, F). Canker lesions progressed in the stems leading to leaf withering at 7 dpi, and at 14 dpi all *D. caulivora*–inoculated plants were dead (Figures 4G, H). Plants treated with PDA were healthy, with no apparent lesions (Figures 4A–D). Development of symptom in leaves was slower than in stems. Detached leaves inoculated with D57 showed necrosis and chlorosis extending from the inoculation site at 5 dpi (Figure 4J). No disease symptoms were visible in control leaves (Figure 4I). Lesions extended in the leaf tissues, and browning of veins was clearly visible (Figures 4K–N). In addition, pycnidia with *a*-conidia, which were hyaline, unicellular, ellipsoid to ovoid (6.5–8.5 × 2.0–3.1), biguttulate, and aseptate, were present in diseased tissues at 10 dpi (Figure 4P). When inoculated leaves were observed in more detail, areas of brown tissues were evident at the base of the trichomes (Supplementary Figure 8). Hyphae stained with solophenyl flavine were associated to the trichomes in leaves and stems. These results suggest that the trichomes could act as physical adhesion points facilitating fungal colonization. Additionally, after ascospores inoculation of petioles, germination occurs and hyphae developed, resulted in browning of the base of the trichomes (Supplementary Figures 10D–F). Browning of petioles or the base of the trichomes were not observed in control tissues (Supplementary Figures 9A–C).

In order to evaluate the aggressiveness of the three selected *D. caulivora* isolates (D47, D57, and D08.4), plants were inoculated by stem wounding, and disease severity was visually assessed at different time points according to the proposed disease scale (Figure 5; Supplementary Figure 2). The results show that disease symptoms were more severe in plants inoculated with D57 at 7 and 14 dpi, compared to D47- and D08.4-inoculated plants (Figure 5A). Lesion length was significantly shorter in stems inoculated with D08.4 at 7, 11, and 14 dpi compared to D47- and D57-inoculated stems (Figure 5B). Significant differences in lesion length between D47- and D57-inoculated tissues were observed at 3, 7, and 14 dpi. Disease severity index increased with time, and was significantly lower at 11 and 14 dpi in soybean plants inoculated with D08.4 compared to D47 and D57 (Figure 5C). The AUDPC was used to compare disease progression in the *D. caulivora*–inoculated plants (Figure 5D). Significant differences in AUDPC values among the three *D. caulivora* isolates were observed; D08.4 = 596.7, D47 = 765.1, and D57 = 915.2. Thus, the results show that D08.4 is the less virulent isolate, while D47 and D57 are more virulent, although difference among D47 and D57 are less pronounced.

Diaporthe caulivora Colonize Rapidly the Vascular Tissue

To gain in-depth knowledge on the infection and colonization process, inoculated stems were further evaluated at early and late time points after inoculation of soybean tissues by *D. caulivora* D57, which grouped together with most isolates. Colonization of *D. caulivora* inside the stem was monitored by confocal microscopy 8, 24, 72, and 96 hpi in transversal sections 1 cm above the wound border. WGA-AF488 was used for fungal cell walls detection, while PI detected plant cell membranes and plant cell walls (Figure 6). In control stem sections no fungal structures were present (Figures 6A–D). At 24 hpi, *D. caulivora* was visualized as green dots which were only detected in the cortex of the stem (Figures 6E–H), and at 48 hpi fungal hyphae increased in the cortex and began to colonize the phloem (Figures 6I–L). At 72 hpi *D. caulivora* was detected in the cortex, phloem, and tracheids and vessels of the xylem (Figures 6M–P). Fungal hyphae were abundant in all vascular tissues at 96 hpi (Figures 6Q–T). Consistently, qPCR showed that fungal biomass started to increase at 8 hpi and at 96 hpi *D. caulivora* DNA became predominant in the stem tissues (Supplementary Figure 10).

At a later time point (7 dpi), the vascular tissues were heavily colonized by *D. caulivora*, as could be observed in transverse and longitudinal sections (Figure 7). Fluorescence associated with the structures of the pathogen was detected towards the edges in the area comprised of vascular bundles (Figures 7B, D, E). Once *D. caulivora* is inside the vascular tissues, it moves throughout the stem, passing from one cell to the adjacent cell (Figures 7D, E). In the cross section, fluorescent points and hyphae were visible, also associated with the vascular bundles (Figure 7B). Differences in browning and thickness of some tissues were observed in *D. caulivora*–inoculated stems compared to control tissues (Figures 7F, G). Stem section of plants inoculated with *D. caulivora* showed necrosis in cortex and secondary phloem, many brown cells in the phloem fibers, and brown cell walls in the vessels. Additionally, the pith softens due to fungal infection and maceration of the tissue, and the cortex becomes smaller and less pronounced compared to control plants, probably due to changes in mechanical properties of the tissues affected by cell collapse and fungal growth. Thus, *D. caulivora* colonizes rapidly

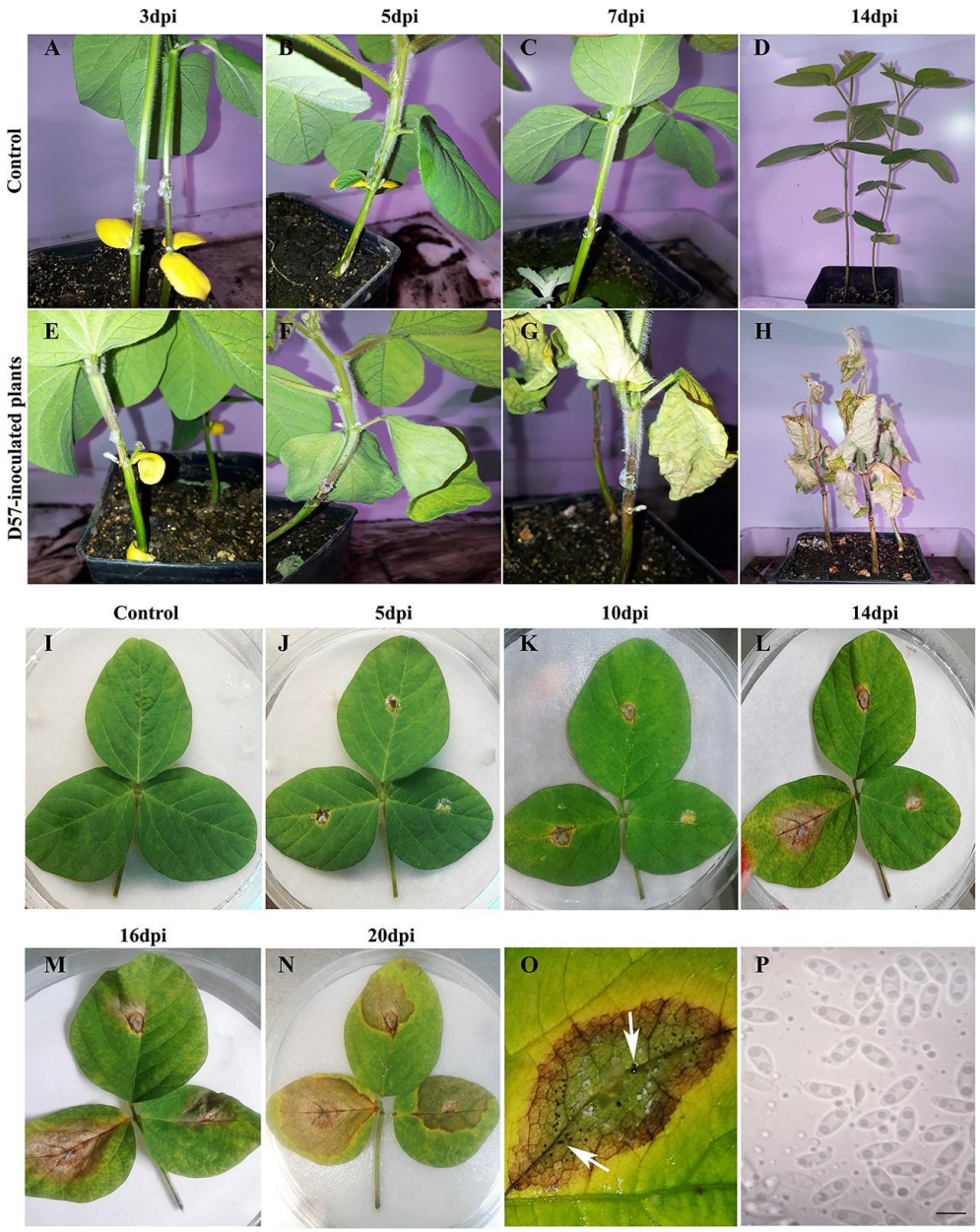
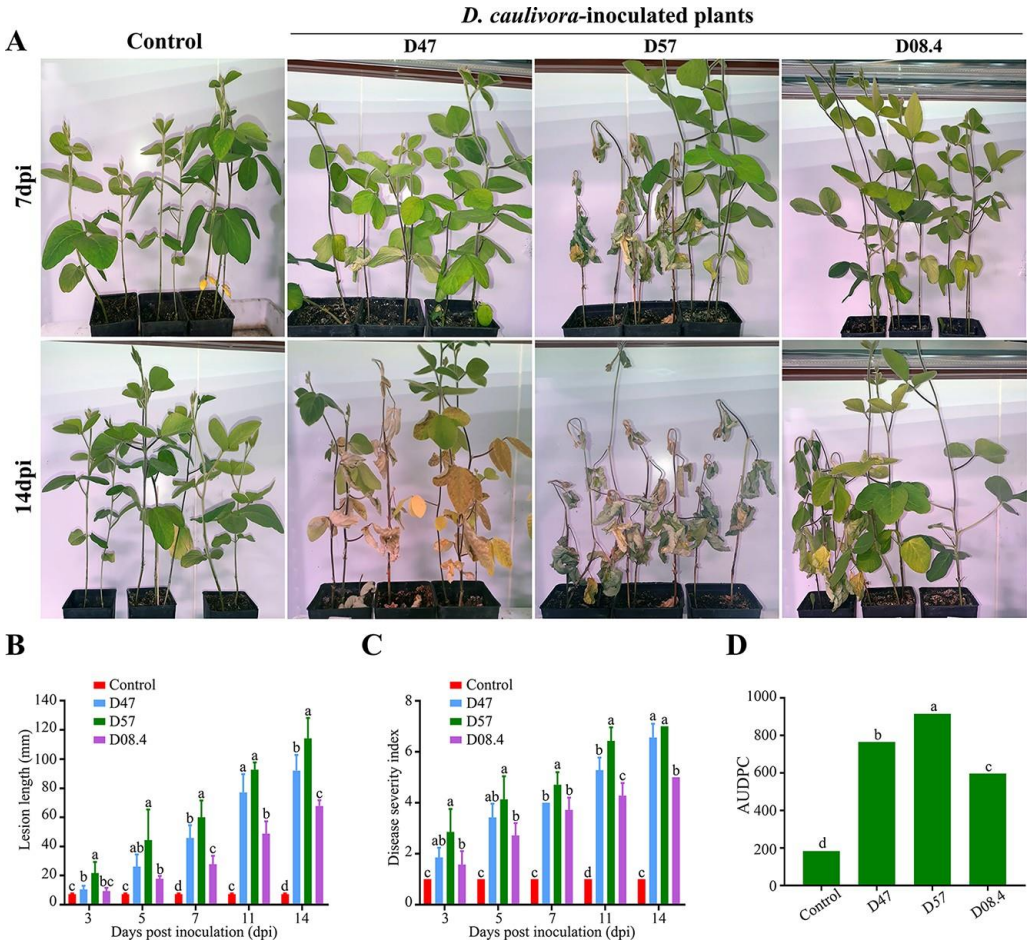


FIGURE 4 | Disease symptoms in tissues infected with *Diaporthe caulivora*. Control PDA treated stems and leaves (A–D, I). Development and evolution of stem canker symptoms on soybean stems (E–H) and leaves (J–N) inoculated with *D. caulivora D57* at different times post-inoculation. Pycnidia present in inoculated leaves [10 days post-inoculation (dpi)] are indicated with an arrow in O. Alpha conidia are shown in P. The scale bar in P represents 5 *m*m.

the vascular bundles leading to browning and softening of the tissues.

D. caulivora Infection Activates Plant Cell Wall Reinforcement

Changes in cell wall composition were observed in *D. caulivora*–infected tissues compared to control tissues after solophenyl flavine staining. In contrast to control tissues, a bright fluorescence was detected in the cell walls of the phloem fibers and all the cells of the xylem of infected stems (Figures 8A, B). Hyphae inside the vessels were clearly visible at 7 dpi (Figure 8B). Changes in cell wall related to defense often include the incorporation of phenolic compounds into the cell walls. We therefore stained the tissue sections with safranin-O



(Figures 8C, D). In control tissues, the phloem fibers and secondary xylem are stained, showing a pinkish-red coloration. Contrarily, all infected tissues were stained with safranin-O, including the cortex and the secondary phloem, showing an intense red-brownish coloration. Moreover, incorporation of phenolic compounds into cell walls of the vessels of the xylem of *D. caulivora*–colonized tissues was also evident by staining. Similarly, *D. caulivora*–infected cortex and secondary phloem changed in color when stained with Toluidine blue, from violet to dark blue, indicating changes in cell wall composition (Figures 8E, F). In addition to the observation that cell wall reinforcement occurs, these three stains evidenced that the cortex and the secondary phloem changes in *D. caulivora*–inoculated stems leading to cellular collapse.

Defense Gene Expression in *D. caulivora*–Inoculated Soybean Stems and Cis-Acting Elements

Expression levels of genes encoding PR-1, PR-2 (*b*-1,3-glucanases), PR-3 and PR-4 (chitinases), PR-10 (Ribonuclease-like protein), LOX2 (lipoxygenase-2), LOX7 (lipoxygenase-7), PDF1.2 (Defensin 1.2), IPER (basic peroxidase), PAL (Phenylalanine-ammonia lyase), and CHS (chalcone synthase) were significantly upregulated in soybean stems inoculated with *D. caulivora* compared to control tissues (Figure 9). Biological significance was only considered when differences in expression values were ≥ 2-fold. Increase in transcript accumulation varied among genes and hours after *D. caulivora* inoculation. *PR-1* expression increased 14-fold after 4 hpi and continued increasing during time, reaching 26,615-fold induction at 48 hpi. *PR-4*, *PR-*

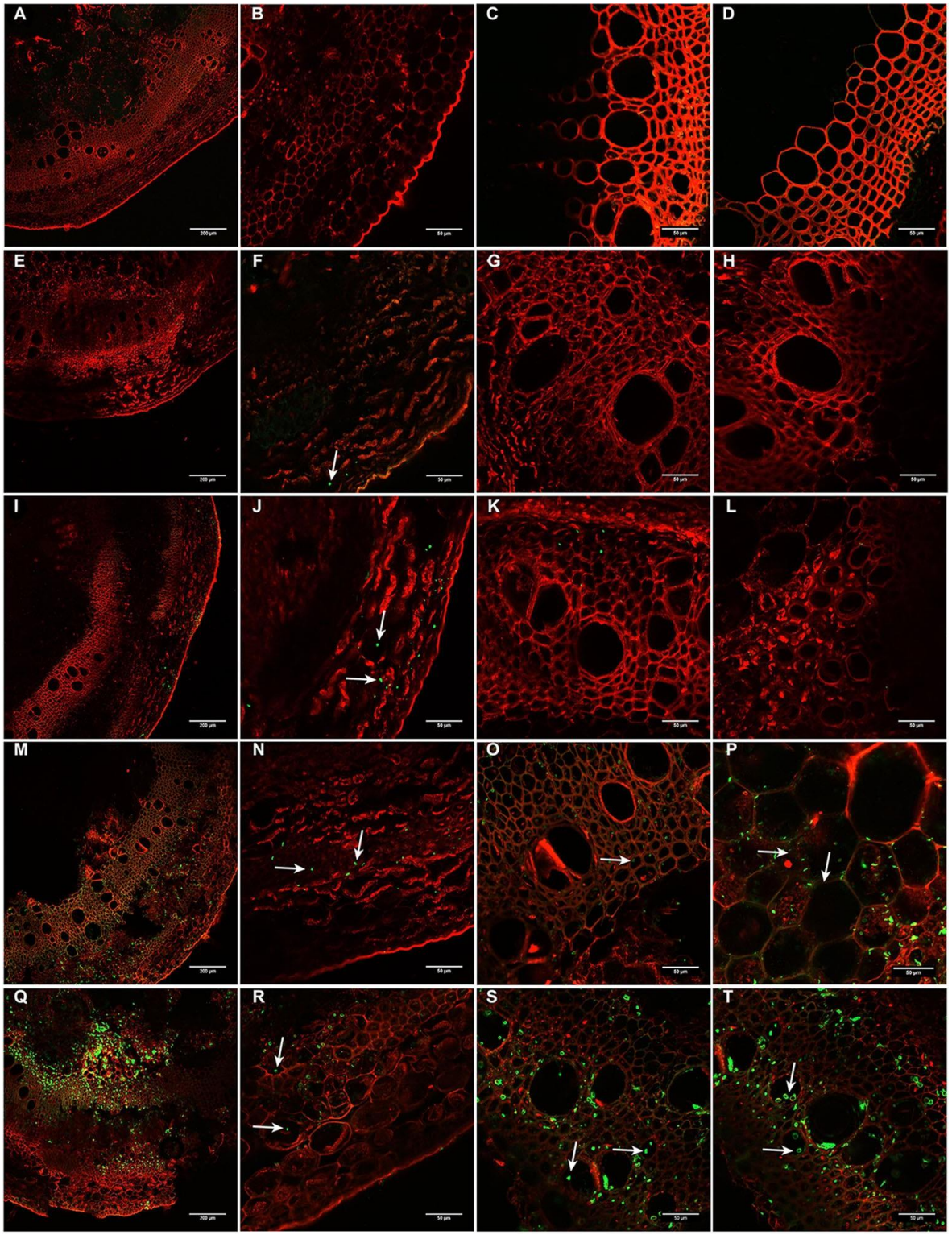


FIGURE 6 | Colonization of *Diaporthe caulivora* in soybean stems at early time points. Wheat Germ Agglutinin–Alexa Fluor 488 conjugate (WGA-AF488) and propidium iodide (PI) were used for fungal cell walls and plant cell membranes/walls detection, respectively in transverse sections. (A–D) non-inoculated plants, (E–H) 24 h post-inoculation (hpi), (I–L) 48 hpi, (M–P) 72 hpi, and (Q–T) 96 hpi. Arrows indicate the localization of *D. caulivora* (isolate D57). Scale bars: 200 *m*m in A, E, I, M, Q, and 50 *m*m in B–D, F–H, J–L, N–P, R–T.

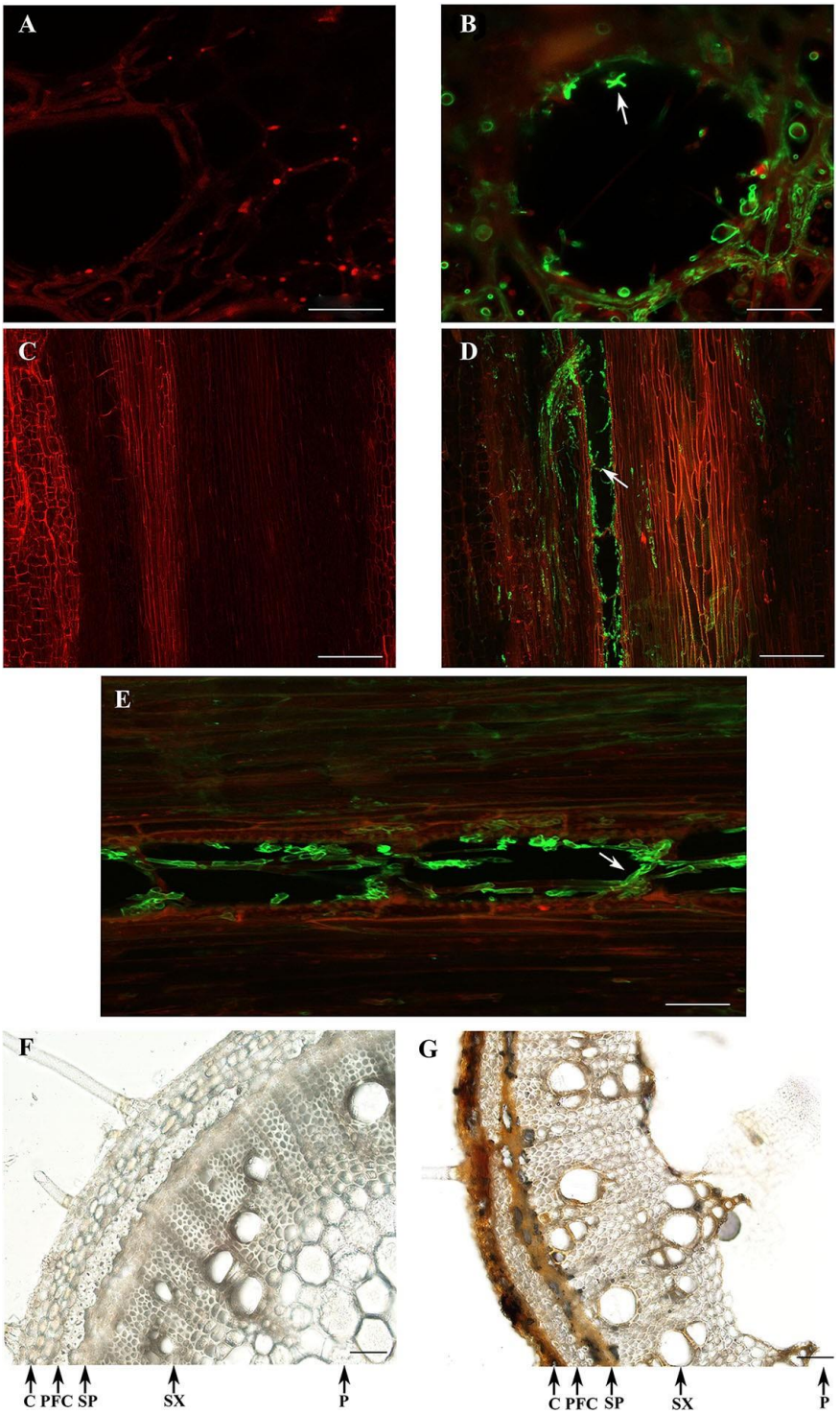


FIGURE 7 | Colonization of *Diaporthe caulivora* in soybean stems at 7 dpi. Wheat Germ Agglutinin–Alexa Fluor 488 conjugate (WGA-AF488) and PI were used for fungal cell walls and plant cell membranes/walls detection, respectively in transverse (A,B), and longitudinal (C–E) sections, and visualized with confocal microscopy. Control tissues (A,C) and *D. caulivora*–inoculated tissues (B,D,E). Arrows indicate the localization of *D. caulivora* (isolate D57). Transversal sections of control (F) and *D. caulivora*–inoculated tissues (G), without staining are also shown. Abbreviations: cortex (C), phloem fibers (PF), secondary phloem (SP), secondary xylem (SX), and pith (P). Scale bars: 20 *m*m in A, B; 200 *m*m in C, D; and 70 *m*m in E, F, G.

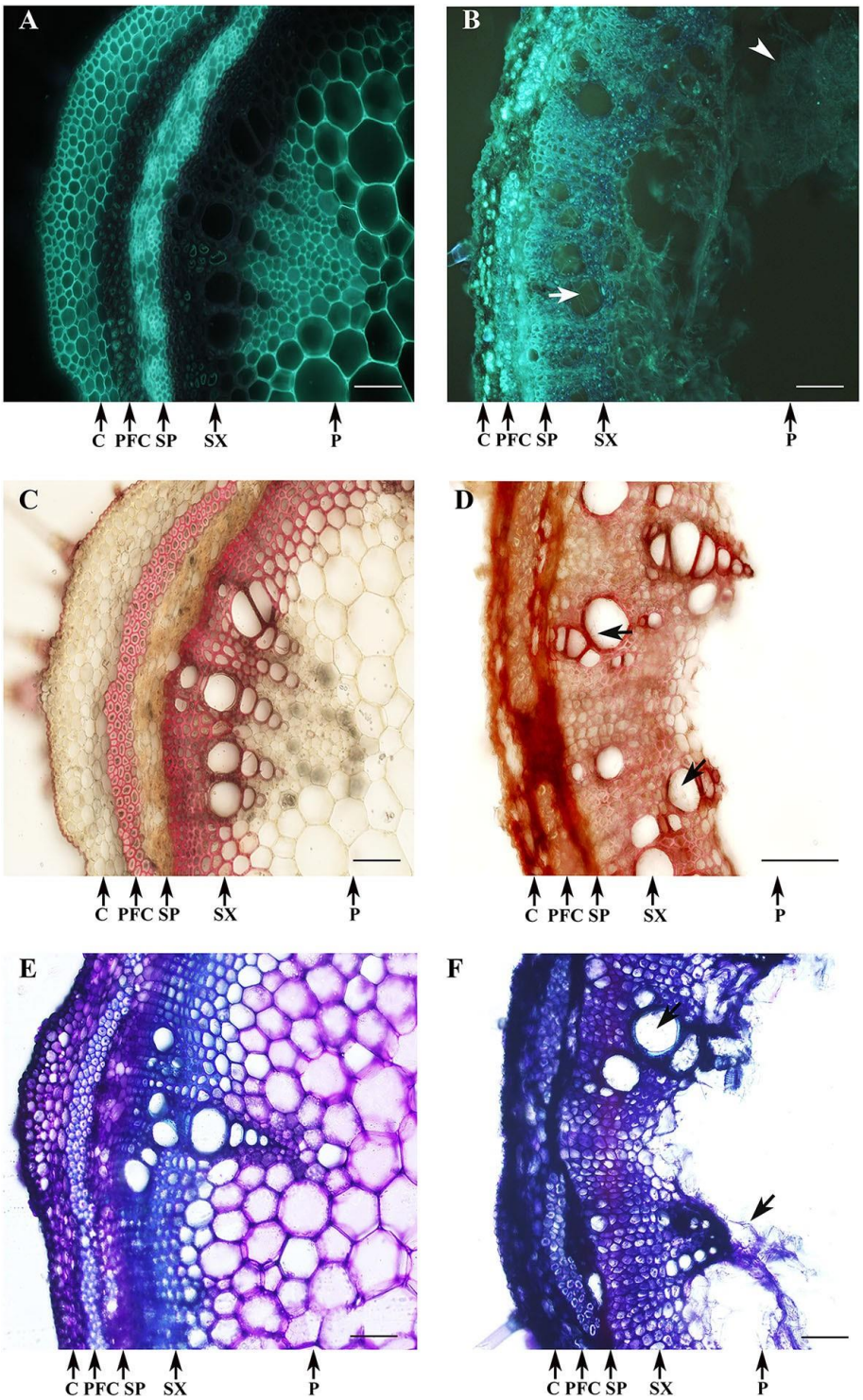
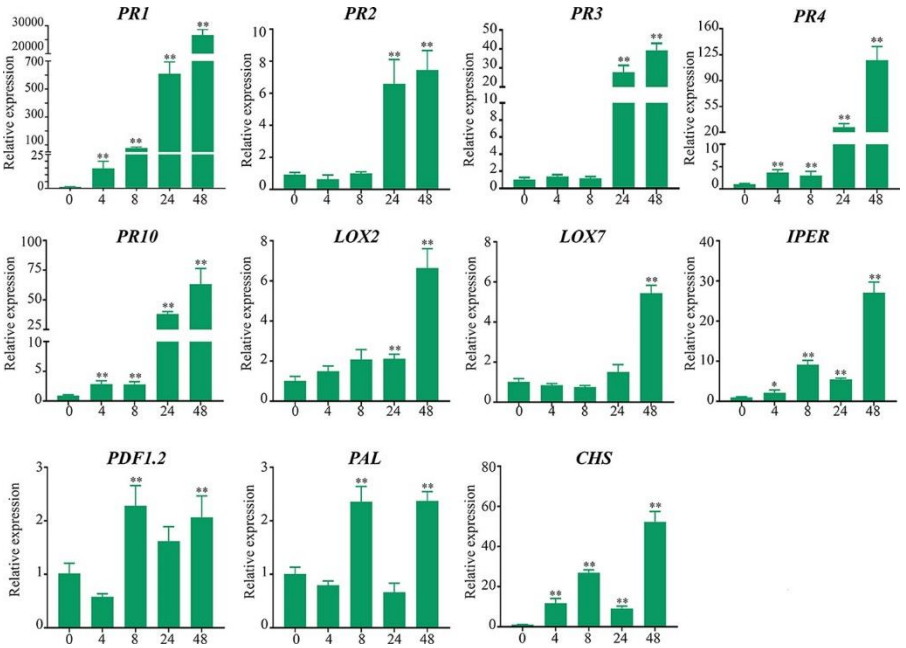


FIGURE 8 | Cell wall reinforcement after *Diaporthe caulivora* colonization. Cell wall–associated defense responses in *D. caulivora*–infected tissues (isolate D57) stained at 7 dpi with solophenyl flavine (A, B), safranin-O (C, D), and Toluidine blue (E, F). PDA-treated stem (A,C,E) and *D. caulivora*–infected stem (B,D,F). Arrows indicates the presence of hyphae in *D. caulivora*–infected vessels. Arrowhead shows heavily infected pith tissues. Abbreviation: phloem fibers caps (PFC). Scale bars represent 70 *m*m.



10, and *IPER* showed similar expression patterns as *PR-1*, although expression levels were lower, reaching maximum of 117-, 64-, and 27-fold, respectively. *PR-2* and *PR-3* expression increased at 24 and 48 hpi compared to control tissues, reaching expression levels of 6- to 7-fold and 27- to 39-fold, respectively. *LOX2* transcript levels increased approximately 2-fold at 24 hpi, and 6-fold at 48 hpi, while *LOX7* increased only at 48 hpi (5-fold). Transcript levels of *PDF1.2* increased only 2-fold. Interestingly, both *PAL* and *CHS* have a biphasic expression profile in response to *D. caulivora*, reaching the highest expression levels at 8 and 48 hpi. Taken together, the results show that different defense genes are highly expressed in stem tissues infected with *D. caulivora*. To gain further insight into the involvement of these genes in defense responses against *D. caulivora*, the promoter regions were analyzed (Supplementary Table 4). Phytohormone responsive elements were present in the promoter region of the 11 defense genes analyzed, including abscisic acid (9 genes), methyl jasmonate (8 genes), ethylene (6 genes), salicylic acid (4 genes), and auxin (2 genes). These are hormones that play important roles in plant defense responses against pathogens, suggesting that they could participate in the soybean defense response against *D. caulivora*.

DISCUSSION

SSC is a disease caused by *Diaporthe* species that affect the production of soybean in South America and other parts of the world. SSC was responsible for soybean yield losses in several countries, including USA, Canada, Argentina, Brazil, Paraguay, Bolivia, and Italy (Backman et al., 1985; Wrather et al., 1997; Allen et al., 2017). Despite the importance of SSC, only few studies on the variability of *Diaporthe* species causing this disease have been performed, and almost no information related to the infection process and the activation of soybean defense mechanisms is available. The identification of the different species associated to SSC is important to understand the characteristics and dynamics of the disease. In this study, we have focused on Uruguayan isolates and compared the results obtained with those available for the region and other soybean- producing areas of different parts of the world. As expected from a previous study (Stewart, 2015), a high proportion of the Uruguayan *Diaporthe* isolates associated to SSC belongs to *D. caulivora* (42%), while 37% were *D. longicolla*, 15% *D. miriciae*, and 5% to *D. kongii/endophytica* and *D. serafiniae*. The high proportion of *D. longicolla* isolates associated to canker lesions was surprising, although we confirmed by inoculation assays that *D. longicolla* produces similar stem lesions as those caused by *D. caulivora*. This is consistent with recent results obtained by Ghimire et al. (2019). *D. longicolla* has been previously isolated from SSC lesions (Gebreil et al., 2015; Mathew et al., 2015); it has been associated to black zone lines on the lower stems of soybean plants (Olson et al., 2015), and it causes soybean stem blight (Cui et al., 2009). *D. longicolla* causes also *Phomopsis* seed decay leading to a reduction in seed germination of up to 90% and seed

mortality (Kmetz et al., 1978; Gleason and Ferriss, 1985). *D. longicolla*, followed by *D. sojae*, are the major fungi of the *Diaporthe/Phomopsis* complex on soybean seeds, while *D. caulivora* and *D. aspalathi* have low seed-borne frequency (Kmetz et al., 1978; Zhang et al., 1999). Twelve isolates were identified as *D. miriciae*, which have also been associated with canker lesions in soybean and mung bean plants (Thompson et al., 2015). One isolate grouped with *D. serafiniae* and *D. infecunda*, and three isolates with *D. kongii* and *D. endophytica*. While *D. serafiniae* has been isolated from sunflower seeds and no reports are available describing disease symptoms in stems, *D. kongii* and *D. endophytica* have been associated to stem cankers in sunflowers in Australia (Thompson et al., 2011; Thompson et al., 2015). Although *D. kongii* and *D. endophytica* have not been correlated to SSC in field studies, *Diaporthe gulyae*, which causes *Phomopsis* stem canker of sunflower, produces reddish-brown lesions leading to plant death in soybean, suggesting that some pathogenic *Diaporthe* species from sunflower can also infect soybean and cause stem disease (Mathew et al., 2017). Consistently, *D. caulivora*, *D. longicolla*, *D. gulyae*, and *D. helianthi* cause stem canker in soybean and sunflower in inoculation assays (Mathew and Markell, 2014). Thus, since several *Diaporthe* species can cause stem lesions (Kontz et al., 2016), a better understanding of the role played by the different pathogens in the development of disease needs further investigation. Moreover, gene transfer between fungi has already been reported (Richards et al., 2011), and a non-pathogenic strains of *Fusarium oxysporum* was converted into pathogenic by the transfer of a chromosome essential for pathogenicity in tomato (Ma et al., 2010). The role played by genetic exchange between different species within the *Phomopsis/Diaporthe* complex in host specificity and pathogenicity remains to be elucidated. Interestingly, 510 potential horizontal gene transfers have been detected in the genome of *D. longicolla*, and 85.3% of them were of fungal origin (Li et al., 2017).

SSC caused by *D. caulivora* was first reported in the USA in the 1970s (Backman et al., 1985), and later it was detected in many other soybean-producing countries including Canada, Italy, the former Yugoslavia, Croatia, and Korea (Jasnić and Vidić, 1985; Zhang et al., 1997; Santos et al., 2011; Sun et al., 2012). In South America, *D. caulivora* was first found in Argentina in 1999 (Pioli et al., 2001), and in 2006 and 2012– 2013, it was identified in diseased plants in Brazil and Uruguay, respectively (Costamilan et al., 2008; Stewart, 2015). In 2002, *D. caulivora* was widely disseminated in the main soybean- producing region of Argentina, where it coexists with *D. aspalathi* (Pioli et al., 2002). At present, *D. caulivora* is considered the predominant pathogen causing SSC in Argentina (Grijalba and Guillin, 2007; Grijalba and Ridao, 2012). The high number of *D. caulivora* isolates in soybean stems with canker lesions also suggests the dissemination of this pathogen in Uruguay. Consistently, no isolate corresponding to *D. aspalathi* was identified probably due to the use of resistant genotypes in Uruguayan farms (Stewart, 2015). In addition, the high presence of *D. caulivora* in the isolates analyzed could also be associated to the lower temperatures present in the southern part of Uruguay since *D. caulivora* and *D. aspalathi* have different temperature preference (Keeling, 1988). Due to the importance of *D. caulivora* as the causal agent of SSC in Uruguay and the region, we selected three genetically different *D. caulivora* isolates (D47, D57, and D08.4) based on ISSR markers. According to growth characteristics, D08.4 could be distinguished from D47 and D57, since D08.4 grows faster in two different media under the same temperature and nutrient availability conditions, including CMA and Czapeck. Morphological characteristics were similar between the three isolates, including asci and ascospores size, which were similar to *D. caulivora* isolates from Argentina and Brazil (Pioli et al., 2001; Costamilan et al., 2008). Variations in the size of perithecial necks were observed between D47 (short), and D57 and D08.4 (long). However, it has been previously shown that this morphological feature is variable and apparently depends on moisture and light conditions (Brayford, 1990; Fernández and Hanlin, 1996). In addition, the three isolates were virulent in the susceptible genotype used, showing typical SSC symptoms. However, isolate D08.4 developed symptoms later, lesions were smaller at 7, 11, and 14 dpi, and it showed the lowest AUDPC values. D47 and D57 were more aggressive than D08.4, and based on visual symptom development and AUPDC values, D57 was the most aggressive isolate. Further studies are needed to evaluate if different races make up the Uruguayan *D. caulivora* population, aspect that could contribute to understand the epidemiology of SSC. Only few studies showing differences in pathogenicity among *D. caulivora* isolates have been performed.

D. caulivora isolates from different origins (Argentina and USA) varied in virulence on different Argentinian susceptible genotypes (Pioli et al., 2003; Benavidez et al., 2010), suggesting that different virulence genes are present in different isolates. More information is available on the pathogenic variability among *D. aspalathi* isolates. Pioli et al. (2003) have shown the existence of variability in virulence of *D. aspalathi* isolates in Argentina, and propose the existence of four different physiological races of *D. aspalathi* based on their interaction with soybean genotypes carrying different SSC resistance genes. Similarly, occurrence of at least three races of *D. aspalathi* based on inoculation on soybean differential set has been identified in Brazil (Brumer et al., 2018). The presence of different *D. aspalathi* races can be due to selection pressure caused by deployment of specific resistant genes (Pioli et al., 1999; Pioli et al., 2003; Brumer et al., 2018). Soybean genotypes carrying Rdm1, Rdm2, Rdm3, and Rdm4 conferred resistance to *D. aspalathi* but not to *D. caulivora* (Pioli et al., 2003). Differences in susceptibility of soybean cultivars to SSC caused by *D. caulivora* have been observed previously (Pioli et al., 2003; Benavidez et al., 2010), and very recently, the first resistance gene, Rdc1, effective against *D. caulivora* was identified (Peruzzo et al., 2019). Further studies are needed to understand the mode of action of *Rdc*1, as well as identifying other resistant genes, which together with the characterization of *D. caulivora* populations could shed light to the development of effective introgression strategies in breeding programs.

D. caulivora colonized leaf and stem tissues, producing necrosis in the stem (canker) with brown vascular tissues

extending from the inoculation site and foliar symptoms with chlorosis surrounding the necrotic areas. Pycnidia were not produced in media or inoculated stem; however, the presence of pycnidia with *a*-conidia was bserved on inoculated leaves. Development of pycnidia in *D. caulivora* is controversial. While pycnidia were not present in Argentinian *D. caulivora* isolates (Pioli et al., 2002), picnidia with *a* and/or *b* conidia were present in isolates of Unites States (Kmetz et al., 1978; Fernández and Hanlin, 1996), Korea (Sun et al., 2012), and Brazil (Brumer et al., 2018). *D. caulivora* hyphae were associated with trichomes in leaves and stems, acting probably as physical adhesion sites of the hyphae. Consistently, trichomes are preferred penetration sites for several fungi, facilitating adhesion of fungal spores and hyphae, and allowing fungal colonization progress on the tissue surface (Lazniewska et al., 2012). Ascospores on infected soybean and crop residue are the main source of inoculum (Grijalba and Ridao, 2012), and our results suggest that hyphae from germinated ascospores could be associated with trichomes. After inoculation, *D. caulivora* started to colonize the cortex at 24 hpi, progressed, and reached the phloem at 48 hpi and the xylem at 72 hpi. At 96 hpi, stem vascular tissues were heavy colonized, which was consistent with high levels of the pathogen quantitation by qPCR and development of canker lesions. Necrosis of the stem cortex was observed at 96 hpi, when symptoms were visually distinguishable. In more advanced stages of *D. caulivora* colonization necrosis of secondary phloem, browning of cells in the phloem fibers and xylem vessels were visible. In addition, changes in infected stems occur, including softening of the pith due to fungal colonization and maceration of the tissue, and the cortex and secondary phloem became smaller and less pronounced compared to control tissues. Other fungal pathogens such as *Phialophora gregata* infect soybean stems and produce similar changes in stem tissues (Impullitti and Malvick, 2014).

Plant cell wall breakdown is one of the first events involved in the infection process of fungal pathogens. These microorganisms degrade the cell walls through the combined action of a wide range of plant cell wall degrading enzymes (PCWDEs), including pectinases, pectate lyases, endopolygalacturonases, cellulases, and cutinases (Bellincampi et al., 2014). A high number of genes encoding PCWDEs have been identified in the sequenced genome of *D. longicolla*, suggesting that they could be important virulence factors (Li et al., 2017). In response to fungal infection plants induce structural defenses, including cell wall reinforcement that limits pathogen colonization of the plant tissues. Increased expression of a soybean gene encoding a protein inhibitor of fungal endopolygalacturonase (PGIP) in plants infected with *D. caulivora* (Favaron et al., 2000) suggests an active plant defense mechanism against the action of PCWDEs. Consistently, we show that *D. caulivora* colonization produces changes in the cell walls that were evidenced with solophenyl flavine staining, which detect xyloglucan (Anderson et al., 2010). Incorporation of phenolic compounds in *D. caulivora*–infected tissues was visible with safranine-O and Toluidine blue staining, suggesting reinforcement of the cell walls. Incorporation of phenolics into cell wall of xylem vessels was evident, which is consistent with the activation of a defense mechanism. Physical defenses which stop or contain the pathogen from further spread in the xylem vessels, as well as chemical defense responses that kill the pathogen or inhibit its growth, have been observed for other vascular pathogens (Yadeta and Thomma, 2013). Vascular wall coating around the infected xylem parenchyma cells and the adjacent xylem vessels has been proposed to prevent lateral and vertical spreading of vascular pathogens in the vessels (Yadeta and Thomma, 2013). Further research is needed to understand how cell wall reinforcement contributes to resistance in different soybean genotypes. RNAseq analysis of contrasting soybean genotypes together with functional studies will allow identifying genes involved in cell wall fortification mechanisms as well as other defense responses that participate in plant resistance against *D. caulivora*, leading to the development of sustainable strategies to control this disease. In addition, genome sequencing of the pathogen will provide important information on the pathogenicity mechanisms that are needed for infection as well as effector molecules that interfere with plant defense.

Activation of defense genes has been associated to basal defense against pathogens in different legumes, including soybean (Samac and Graham, 2007). Here, we observed that expression of several defense genes was upregulated in stem tissues infected with *D. caulivora* compared to control tissues. The highest transcript accumulation in *D. caulivora*–inoculated tissues compared to control tissues was observed for *PR-1*. Such high *PR-1* expression levels have been previously observed in pathogen infected *Arabidopsis* tissues (Inada et al., 2016). Genes encoding a *b*-1,3-glucanase (PR-2), two chitinases (PR-3 and PR- 4), and a PR-10 were also highly induced after *D. caulivora* inoculation. These PRs have antimicrobial activities and have been associated to activation of defenses against different pathogen in soybean (Moy et al., 2004; Zou et al., 2005; Upchurch and Ramirez, 2010; Schneider et al., 2011). *PR1*, *PR2*, *PR3*, *PR4*, *PR5*, and *PR10* are constitutively expressed in soybean plants overexpressing NPR1 (nonexpressor of PR-1), leading to increased resistance to *P. sojae* (Fan et al., 2017). Similarly, *PR10* overexpression increases resistance against *P. sojae* (Fan et al., 2015). Here, we show that a gene encoding a basic peroxidase (IPER) is rapidly induced in stem tissues infected with *D. caulivora*, and transcript continued accumulating during fungal colonization. PR1, PR-2, and IPER are proposed to contribute to partial resistance to *P. sojae* in soybean (Vega-Sánchez et al., 2005). In addition, peroxidases and lipoxygenases (LOXs), PRs, and genes involved in the phenylpropanoid pathway are expressed in soybean tissues infected with *P. pachyrhizi* (Schneider et al., 2011). LOXs participate in the biosynthetic pathways leading to the production of different oxylipins with diverse roles in defense, including the hormone jasmonic acid (Blée, 2012). Expression levels of two *LOXs* genes are induced by *D. caulivora*, suggesting that oxylipins could play a role in defense against this fungus. *PAL* and *CHS* transcript levels also increases after *D. caulivora* inoculation. Genes encoding enzymes in the phenylpropanoid pathway, including PALs and CHSs, were also upregulated in

response to *Pseudomonas syringae* and *P. sojae* in soybean (Moy et al., 2004; Zabala et al., 2006). The phenylpropanoid pathway leads to the production of compounds, which play different roles in defense, including flavonoids, coumarins, and lignans, and in soybean the reduction of flavonoids by an RNAi approach resulted in enhanced susceptibility to *P. sojae* (Subramanian et al., 2005). Expression analysis of defense genes after *D. aspalathi* infection has been performed in leaves and seeds (Upchurch and Ramirez, 2010). In response to *D. aspalathi PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR10*, and *LOX7* are induced in leaves. In seeds, transcript levels of *PR-1*, *PR-2*, *CHS*, *PAL*, and *IPER* increased after *D. aspalathi* inoculation. Thus, several of these genes are important for defense responses against both *D. caulivora* and *D. aspalathi* pathogens. Interestingly, *PR-1*, *PR-5*, several *PALs*, and *CHS* expression levels are higher expressed in resistant compared to susceptible soybean genotypes after *Fusarium solani* f. sp. *glycines* infection (Iqbal et al., 2005). Similarly, transcript levels of genes encoding peroxidases and LOXs were higher in a resistant interaction of soybean with *P. pachyrhizi* compared to a susceptible interaction (Choi et al., 2008). Thus, several of these genes are important in the defense response of soybean to different pathogens, including *D. caulivora*. Phytohormones including jasmonic acid, salicylic acid, ethylene, abscisic acid, and auxin play important roles in the defense response of plants against different pathogens (Denancé et al., 2013). Here we show that all the defense genes analyzed, which are *D. caulivora*– inducible, have cis-acting regulatory element in their promoters involved in phytohormone responsiveness. Interestingly, most genes have cis-acting elements involved in abscisic acid responsiveness, and several contain elements related to methyl jasmonate, ethylene, and salicylic acid responsiveness. Further analyses are required to reveal the involvement of these hormones in the defense response against *D. caulivora*. The availability of the complete soybean genome, the presence of soybean genotypes contrasting for resistance to *D. caulivora*, and the increasing number of resources for functional genomics will help to identify key components in the plant defense response and to design strategies to enhance resistance to this important pathogen.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GeneBank database (MK483139-MK483213, MK507892, and MN584748-MN584826).

AUTHOR CONTRIBUTIONS

EM performed all the experiments and helped to write the manuscript. SS interpreted the data, contributed to discussions, and helped to write the manuscript. MM helped to design and to supervise the study, interpreted the data, contributed to discussions, and helped to write the manuscript. IP designed and supervised the study, interpreted the data, and wrote the manuscript. All authors read and approved the final 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/fpls.2019.01733/full#supplementary-material

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Capítulo II

Estudios de genómica comparativa de especies de *Diaporthe* patógenas de plantas, y análisis transcriptómicos de *Diaporthe caulivora* durante la infección, evidencian estrategias de patogenicidad del género

El género *Diaporthe* ha sido estudiado en diferentes patosistemas. Hasta la fecha hay varios genomas secuenciados de especies patógenas como *D. capsici, D. citri, D. destruens, D. longicolla, D. phragmitis, D. helianthi, D. ampelina, D. aspalathi, D. citriasiana, D. citrichinensis* (Li *et al*., 2015; Morales-Cruz *et al*., 2015; Baroncelli *et al*., 2016, Savitha *et al*., 2016; Li *et al*., 2017; Fang *et al*., 2020; Liu *et al*., 2021, Huang *et al*., 2021; Wang *et al*., 2021; Gai *et al*., 2021)

En esta parte del trabajo secuenciamos y anotamos por primera vez el genoma de *D. caulivora* (D57) mediante tecnología PacBio con una cobertura de 270X. Se generaron secuencias largas de buena calidad agrupadas en 10 contings y con un tamaño estimado de 57.86 Mb. Este tamaño de genoma es similar al encontrado para otros genomas del género los cuales van desde 56.1 hasta 64.7 Mb (Li *et al*., 2015; Fang *et al*., 2020; Liu *et al*., 2021, Huang *et al*., 2021; Wang *et al*., 2021). El ensamblado está entre los mejores obtenidos hasta la fecha para *Diaporthe* teniendo en cuenta la cantidad de N, la completitud según BUSCO y la relación entre el número de contings y el largo de los mismos. Se identificaron 18.385 genes que codifican proteínas y cuyos transcriptos están presentes en los transcriptomas que se utilizaron para la anotación.

Las proteínas secretadas median la comunicación con el hospedero y están relacionadas con la patogénesis (McCotter y Horianopoulos, 2016). Los efectores son pequeñas moléculas secretadas por el patógeno que manipulan las células del hospedero facilitando la infección y evadiendo la respuesta de defensa (Toruño *et al*., 2016). Con el fin de conocer componentes moleculares de este fitopatógeno, se identificaron 1598 posibles efectores siendo 1445 de ellos citoplasmáticos y 150 apoplásticos. También se analizaron 1501 genes que codifican proteínas secretadas por *D. caulivora*.

Además, nos enfocamos en los genes que codifican proteínas secretadas de las cuales 460 codifican enzimas que degradan carbohidratos (CAZymes), 556 fueron identificadas en la base de datos Pathogen host interaction (PHI-base), que contiene datos funcionales en diferentes patógenos de plantas, y 133 codifican para efectores candidatos. En el PHI-base 287 estan relacionadas con la reducción de virulencia, 18 la incrementaron y 35 han sido descritos como efectores. La gran proporción de proteínas secretadas está en relación con modificaciones de la pared celular, lo cual desde el punto de vista del patógeno facilita la infección y su nutrición (Zhao *et al*., 2013).

Además, realizamos un estudio comparativo con cinco de los genomas disponibles de otros *Diaporthe* fitopatógenos en otros hospederos (*D. capsici, D. citri, D. destruens, D. longicola, D. phragmitis*). En este estudio hicimos énfasis en los genes que compartían con otros hongos patógenos, los que compartían entre sí como especies del mismo género *Diaporthe* y los que eran específicos de *D. caulivora*. En total 1375 proteínas predichas tenían homólogos en otros *Diaporthe* y patógenos fúngicos. Entre las seis especies de *Diaporthe* 439 proteínas secretadas son compartidas e incluyen proteínas relacionadas con la degradación y modificación de la pared celular como pectinas, hidrolasas, endo y exoglucanasas, proteasas, peptidasas, lipasas, peroxidasas entre otras. Además 46 proteínas son específicas de *Diaporthe* y 27 específicas de *D. caulivora*.

Al comparar el número de genes que codifican enzimas que degradan carbohidratos, las seis especies de *Diaporthe* presentaron 424-488 genes, de los cuales 156 eran comunes en todas las especies y entre 331-382 eran compartidos con respecto a *D. caulivora*. Respecto al número de efectores el rango entre los *Diaporthe* fue de 85-133, incluyendo liasas, hidrolasas, proteínas relacionadas con la patogénesis, peptidasas, esterasas y otras. La mayoría de los efectores están presentes en *Diaporthe* y otros hongos patógenos, 9 efectores están presentes en todos los *Diaporthe* y 4 son específicos de *D. caulivora*.

Generamos conocimiento de la interacción a partir del análisis de los transcriptomas de *D. caulivora* los primeros estadíos de infección (8 y 48 hpi) de plantas de soja, siendo el primero de estos estudios para el género *Diaporthe*. Se obtuvieron un total de 2659 genes diferencialmente expresados (DEGs, del inglés Differential expresion genes), la mayoría de ellos a 48 hpi. 119 de los DEGs están involucrados en la patogenicidad de *D. caulivora*. El patrón de expresión de los genes inducidos y el análisis del enriquecimiento evidenció que las estrategias de infección de *D. caulivora* están relacionadas con la degradación y modificación de la pared celular, la detoxificación de compuestos, la actividad de transportadores y la producción de toxinas. Encontramos inducción de varios genes que codifican para oxidorreductasas, dehidrogenasas y peroxidasas, las cuales han sido descritas previamente por su rol en la colonización de hongos y el mantenimiento del estado redox (Segal y Wilson, 2018; Abdul *et al*., 2018). También se inducen varios genes involucrados en la síntesis de toxinas fúngicas, proteínas que producen necrosis e inducción de etileno y poliquetido sintasas, las cuales cumplen roles importantes en la patogenicidad de una variedad de patógenos fúngicos (Ruocco *et al*., 2018). La inducción de genes que codifican para transportadores es consistente con la exportación de compuestos involucrados en la patogénesis, así como en la secresión de compuestos con actividad antimicrobiana (Morrissey y Osbourn, 1999; Coleman y Mylonakis, 2009).

Según nuestra revisión bibliográfica, este sería el primer estudio que incluye la comparación entre especies del género *Diaporthe*, así como la primera secuenciación y anotación del genoma de *D. caulivora* y el primer estudio de transcriptoma de *Diaporthe*

en plantas. Los resultados obtenidos nos permitieron concluir que el genoma y los mecanismos de patogenicidad son similares, independientemente de que tengan diferentes hospederos. Por otro lado, resulta interesante destacar que cada especie tiene grupos de genes especie-específicos, algunos de los cuales pueden estar involucrados en la patogénesis, así como en interferir con la respuesta de defensa de la planta. El análisis completo combinado del genoma y transcriptoma brinda nueva e importante información sobre los mecanismos moleculares implicados en la patogénesis de *D. caulivora* y el proceso de colonización del hospedero.

Comparative genomics of plant pathogenic *Diaporthe* species and transcriptomics of *Diaporthe caulivora* during host infection reveal insights into pathogenic strategies of the genus

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Abstract

Background: *Diaporthe caulivora* is a fungal pathogen causing stem canker in soybean worldwide. The generation of genomic and transcriptomic information of this ascomycete, together with a comparative genomic approach with other pathogens of this genus, will contribute to get insights into the molecular basis of pathogenicity strategies used by *D. caulivora* and other *Diaporthe* species.

Results: In the present work, the nuclear genome of *D. caulivora* isolate (D57) was resolved, and a comprehensive annotation based on expression evidence and genomic analysis is provided. This genome

has an estimated size of 57,86 Mb and contains 18,385 predicted protein-coding genes, from which 1501 encode predicted secreted proteins. A large array of *D. caulivora* genes encoding secreted pathogenicity-related proteins was identified, including carbohydrate-active enzymes (CAZymes), necrosis-inducing proteins, oxidoreductases, proteases and effector candidates. Comparative genomics with other plant pathogenic *Diaporthe* species revealed a core secretome present in all *Diaporthe* species as well as *Diaporthe*-specific and *D. caulivora*-specific secreted proteins. Transcriptional profiling during early soybean infection stages showed differential expression of 2659 *D. caulivora* genes. Expression patterns of upregulated genes and gene ontology enrichment analysis revealed that host infection strategies depends on plant cell wall degradation and modification, detoxification of compounds, transporter activities and toxin production. Increased expression of effectors candidates suggests that *D. caulivora* pathogenicity also rely on plant defense evasion. A high proportion of the upregulated genes correspond to the core secretome and are represented in the pathogen-host interaction (PHI) database, which is consistent with their potential roles in pathogenic strategies of the genus *Diaporthe*.

Conclusions: Our findings give novel and relevant insights into the molecular players involved in pathogenicity of *D. caulivora* towards soybean plants, several of which are in common with other *Diaporthe* pathogens with different host specificity, while others are species-specific. Our analyses also highlight the importance to have a deeper understanding of pathogenicity functions among *Diaporthe* pathogens and their interference with plant defense activation.

Keywords

Diaporthe caulivora, soybean, *Diaporthe* pathogens, genome, comparative genomics, RNAseq, pathogenicity factors, secretome, effectors

Background

Species of the genus *Diaporthe* and their anamorph *Phomopsis* states are endophytes, pathogenic, and saprophytic on a wide range of hosts worldwide [1-3]. Pathogenic *Diaporthe* species cause diseases on a wide range of plants hosts, including forest trees [4], citrus [5], pepper [6], sunflower [7], and soybean [8],

among others. In soybean, *Diaporthe caulivora* (syn. *Diaporthe phaseolorum* var. *caulivora*) and *Diaporthe aspalathi* (syn. *Diaporthe phaseolorum* var. *meridionalis*) are the causal agents of soybean stem canker (SSC) [8-11]. These pathogens are often associated with other species of the same genus, including *Diaporthe longicolla*, which also cause the disease [12-13]. SSC causes important losses in soybean growing regions around the world [14-18], and disease symptoms are associated principally to necrosis of the stem, with discoloration and leaf wilting [10,13]. Currently, SSC management is performed by agronomic practices (crop rotation, avoidance of crop residue), fungicide applications, and the use of resistant cultivars [12,19]. Host-plant resistance is currently the most effective strategy and therefore the identification of sources of resistance in soybean germplasms and breeding lines is of great importance. Until present, five major genes that confer resistance to SSC caused by *D. aspalathi* and one for *D. caulivora* have been identified [10,20-21]. However, no commercial varieties with resistance to *D. caulivora* are available. Since *D. caulivora* is one of the principal causal agent of SSC in soybean producing countries [13,19,22), more information is needed to understand the interaction of this pathogen with the host plant in order to develop breeding strategies to control the disease.

Genomic information has allowed the identification of virulence factors and effector proteins in different pathogenic fungal species [23-24], including plant cell wall degrading enzymes (PCWDEs) and enzymes involved in toxin production that are important for host colonization [25-27]. At present, 12 nuclear genome sequences of *Diaporthe* species are available at NCBI [6,26,28-37]. However, genomic resources of the important pathogen *D. caulivora* are not available and transcriptomic profiling of *Diaporthe* species during host infection has not been performed. The generation of this information will give insights into the molecular basis of the pathogenicity strategies used by different *Diaporthe* species. In the present work, we report for the first time the genome sequence of *D. caulivora* and performed a comparative genomics study with five previously available pathogenic *Diaporthe* genome sequences with different host range [6,13,35-41]. Moreover, we present RNAseq data of *D. caulivora* during host plant infection. Our findings reveal the presence of pathogenicity factors that are shared between all *Diaporthe* species and *D. caulivora*-specific virulence components involved in host colonization and plant defense evasion.

Results and discussion

Diaporthe caulivora de novo genome sequencing, assembly and comparison with available *Diaporthe* genomes

The nuclear genome assembly of *D. caulivora* was resolved taking the advantages of PacBio sequences reads using FlyE v2.7. The assembly consisted in 10 contigs with a total length of 57,864,239 bp and a coverage of 270X (**Table 1**). The polishing step was performed using Minimap2 v2.18 by mapping the Pacbio raw reads back to the genome assembly [42], with a mapping rate of 97.8%. Genome assembly and the annotation was defined with high precision and completeness, determined by BUSCO analysis; 98% completeness in fungi_odb10.2019-11-20 database. We further looked for nuclear genomes of other *Diaporthe* species available at NCBI and selected five genomes based on the quality of the assembly (**Additional File 1**, **Table 1**). Although *D. longicolla* has a lower quality assembly, it was included since this species has high occurrence in SCC lesions [13]. The other selected *Diaporthe* species were all pathogens with different host range (**Additional File 2**). The genome assembly sizes of *D. capsici, D. destruens* and *D. phagmatis* ranged from 56,1 Mb to 58,3 Mb [6,36-37], and were comparable in size with our 57,86 Mb *D. caulivora* genome sequence, while *D. longicolla* and *D. citri* were ~ 7 Mb larger than the other species analyzed [26,35] (**Table 1**).

Table 1: General features of *Diaporthe* genomes

***Diaporthe* species**	***D. caulivora***	***D. capsici***	***D. citri***	***D. destruens***	***D. longicolla***	***D. phragmitis***
Total genome size (bp)	57864239	57558510	63685968	56108228	64714568	58328132
Coverage (X)	270	ND	271	121	145	50
Contigs	10	20	34	47	985	28
Maximum contig length (bp)	14464108	8755198	12370252	6293594	1124325	7711659
N50 (bp)	8708519	5171887	5472022	2479481	204364	3550333
GC(%)	52.97	51.27	46.72	48.7	48.26	50.82
Complete BUSCO (%)	97.8	ND	98.5	97.93	98.21	97.9
Duplicated BUSCO (%)	0.6	ND	ND	3.33	ND	1
Fragmented BUSCO (%)	0.43	ND	ND	ND	ND	ND
Missing BUSCO (%)	1	ND	ND	ND	ND	1.1
Protein-coding gene number	18385	14425	15921	13754	16606	12393
Total gene length (bp)	23421216	23205508	26007773	ND	28344980	16320211

Average gene length (bp)	1690	1609	1633.5	ND	1709	1317
Protein-coding gene number (Augustus with *D. caulivora*)*	18385	15675	15113	13717	15232	15655
Predicted secretome *	1501	1588	1383	1298	1535	1539
Predicted effectors >80% probability*	1598	1204	1088	1103	1229	1168
Cytoplasmic effectors*	1448	1043	938	982	1073	1009
Apoplastic effectors*	150	161	150	121	156	159
Genbank accesion no.	BioProject PRJNA717308	WNXA00000000	JACTAD000000000	JACAAM000000000	JUJX00000000	JACDXY000000000
References	this paper	[6]	[34]	[36]	[28]	[37]

ND: no data
* data from this paper

A whole genome nucleotide alignment using MUMmer [43], implemented in SyMap V 5.0.6, allowed the identification of large regions in syntenic blocks between *D. caulivora* and the other five *Diaporthe* species with an average of 96% (**Additional File 3**). This analysis showed that *D. caulivora* genome has a range of 82 to 92 syntenic blocks with a coverage of 95% of the total length of the genomes respect to *D. capsici*, *D. citri*, *D. destruens* and *D. phragmatis* genomes. It is worth noting that *D. caulivora* contig 10 has a complete synteny with contig 19 of *D. capsici*. The other contigs of *D. caulivora* were highly conserved with a few rearrangement compared with the four *Diaporthe* species mentioned above. The *D. longicolla* assembly was the only one obtained only by Illumina technology and showed the lowest values of synteny, which is consistent with the degree of fragmentation of the assembly.

Gene prediction, functional annotation and orthologs

The annotation of the *D. caulivora* genome assembly was performed using a Fungap pipeline with combined abinitio strategies, homology-based searches and RNA-seq data, resulting in the annotation of 18,385 protein-coding regions. The functional annotation of predicted protein-coding genes was completed with the Blast2GO (B2Go) program [44]. The predicted genes were compared by BlastP against NCBI nr database (TaxId: *Fungi*) and classified by InterProScan v.5.19., Pfam protein families, InterPro domains, Gene Ontology Classification (GO), and metabolic pathways were recovered from proteins identified by BLAST using the B2Go annotation system (**Additional File 4**). Functional description could be assigned to 16,068 coding genes

(87.4%). The remaining 2,317 genes were searched at NCBI and 1787 of them did not show any hit and correspond to putative *D. caulivora*-specific genes validated by transcriptomic data (**Additional File 4**). In order to make comparisons at gene levels with the five genomics assemblages of the other *Diaporthe* species included in this work, we performed gene prediction of the *Diaporthe* genomes with the Augustus web server (http://bioinf.uni-greifswald.de/webaugustus/), using *D. caulivora* protein-coding gene models as a training set. The total predicted coding genes were 15,675 genes models for *D. capsici*, 15,921 for *D. citri*, 13,754 for *D. destruens,* 16,606 for *D. longicolla*, and 12,393 for *D. phragmitis* (**Table 1**).

OrthoFinder analysis combined with an all-versus-all protein BLAST strategy was used to cluster protein orthologous groups and infer a phylogeny tree with the six *Diaporthe* species and *Fusarium graminearum* as outgroup (**Figure 1**). The results show that an overall 94% (86% - 97%) of genes were assigned to orthologous groups shared by the six *Diaporthe* species. Phylogenetic analysis showed that the six *Diaporthe* species were divided into two main clusters (**Figure 1A**). *D. caulivora* was the more distant species included in the first cluster, which could be explained by the lower number of orthologous genes (87%) shared with the other three species. *D. capsici*, *D. citri* and *D. phragmatis*, the closest species, share an average of 98,4% of the orthologous genes, which is in agreement with what has been shown by Gai et al [35] regarding *D. citri* and *D. capsici* phylogenetic relationship. The second cluster included *D. destruens* and *D. longicolla.* Although *D. caulivora* and *D. longicolla* are soybean pathogens and they could be expected to be more closer related, *D. longicolla* shares a similar number of genes assigned to orthologous groups with the other *Diaporthe* species regardless of host specificity (**Figure 1B**). Taking into account the quality of the analyzed assemblages, it is possible that specific genes of each species have not been fully recovered, unlike the *D. caulivora* genome presented in this work. Besides identifying the orthologous genes, we analyzed the average nucleotide identity (ANIm) of the species, which measure the nucleotide-genomic similarity between two genomes. The ANIm values between the different *Diaporthe* species varied from 78,6 to 96,4% and similar clusters as those obtained by orthologous analyses were obtained, except for *D. caulivora* which grouped together with *D. longicolla* and *D. destruens* (**Figure 1C**).

***D. caulivora*-specific and shared *Diaporthe* virulence components**

Fungal secreted proteins mediate communication with the environment including host plants, and have important function in pathogenesis [45]. To define the set of proteins considered within the secretome of the six *Diaporthe* species analyzed in our work, we used SignalP v5.0, WoLF PSORT for Fungi and Phobius. *D. caulivora* has a total of 1501 genes encoding predicted secreted proteins. The predicted secretome of *D. capsici*, *D. longicolla* and *D. phragmitis* was similar in number (ranging from 1588 to 1535 proteins), while *D. citri* and *D. destruens* have a smaller secretome, represented by 1383 and 1298 proteins, respectively (**Figure 2A; Additional File 5**). *D. caulivora* shared between 1007 and 1208 secreted proteins with the other five *Diaporthe* species (**Figure 2B**).

In total 439 secreted proteins were in common between all *Diaporthe* species and were defined as the core secretome (**Figure 3A; Additional File 5**). This core secretome includes virulence components related to plant cell wall degradation and modification, such as pectin and pectate lyases, glycoside hydrolases, carbohydrate esterases, endoglucanases and exoglucanases, a xylanase, as well as proteases, peptidases, lipases, peroxidases, among others. Combining the results of the six secretomes with a NCBI search that include recently sequenced *Diaporthe* and other fungal species, we identified 1375 *D. caulivora* predicted proteins that have conserved homologs in other *Diaporthe* and fungal pathogens (**Figure 3B, Additional File 6**). The rest comprised 53 conserved proteins among *D. caulivora* and other fungal species, 46 *Diaporthe*-specific proteins and 27 *D. caulivora*-specific proteins that exhibited no hit with any other organism. Interestingly, five of the *Diaporthe*-specific proteins belong to the core secretome and represent relevant secreted proteins in the *Diaporthe* genus. *Diaporthe*-specific and *D. caulivora*-specific secreted proteins were all uncharacterized proteins whose roles in pathogenesis needs further investigation.

We looked into more detail to CAZYmes present in the *D. caulivora* secretome, and identified a total of 460 genes encoding CAZymes (**Figure 4**; **Additional File7**). The high proportion of CAZymes in the *D. caulivora* secretome (30,6%), is consistent with previous reports in other plant pathogens and *Diaporthe* species and emphasizes the importance to degrade efficiently polysaccharide materials present in the plant cell walls to facilitate infection and/or gain nutrition [46, 26, 29]. The other *Diaporthe* species have a similarly proportion of their secretome represented by CAZymes; 488 in *D. capsici* (30,7%), 425 in *D. citri* (30,7%), 424 in *D. destruens* (32,7%), 482 in *D. longicolla* (31,4%), and 485 in *D. phragmitis* (31,5%). The diversity of sub-

categories of CAZymes, including Glycoside Hydrolase (GHs), Glycosyltransferase (GTs), Polysaccharide Lyase (PLs), Carbohydrate Esterase (CEs), were similar among the six *Diaporthe* species (**Figure 4A**). The number of shared secreted CAZymes between *D. caulivora* and the other *Diaporthe* species varied between 331 and 382 (**Figure 4B**), and 156 CAZymes were present in all species (**Additional File 7**). These findings suggest that secreted CAZymes play similar roles among *Diaporthe* species in terms of carbon utilization capabilities.

We further search in the Pathogen–Host Interaction (PHI)-base (**Figure 3A, Additional File 8**), which catalogs experimentally verified pathogenicity, virulence and effector genes from different plant pathogens [47]. In total, 556 secreted proteins of *D. caulivora* (37%), were identified in the PHI-base and among them, 287 were related to reduced virulence or loss of pathogenicity mutant phenotypes, 18 to increased virulence and 35 to effectors (**Additional File 8**). From them, 191 belongs to the core secretome and half of them correspond to CAZymes reinforcing their important role in *Diaporthe* pathogenicity (**Figure 3A**). Interestingly, several *D. caulivora* secreted proteins that are shared with other pathogenic fungi but were absent in available genomes of *Diaporthe* species include CAZymes, a kievitone hydratase involved in phytoalexin detoxification [48], several FAD-binding domain-containing proteins, a putative versicolorin B synthase involved in mycotoxin production [49], among others (**Additional File 6**). Five of these *D. caulivora* secreted proteins, represent homologs of fungal genes that were assigned as effectors or result in reduced virulence in knockout or mutant experiments, including two LysM domain-containing proteins, an endo-beta-1,6-glucanase, a pectin lyase-like protein, and a minor extracellular protease vpr (**Additional File 8**). Moreover, several genes encoding important virulence proteins in other fungi, such as necrosis- and ethylene-inducing proteins (NEP), transporters, oxidoreductases, proteases, hypersensitive response-inducing proteins, CFEM domain-containing protein were also present in the secretomes of several of the analyzed *Diaporthe* species, and most of them have PHI-base accessions hits.

Effectors are proteins or small molecules secreted by pathogens, which manipulate host cells, facilitating infection and interfering with host immunity [23,50]. We identified 133 secreted *D. caulivora* effector candidates (**Figure 2A**), including pectate and other polysaccharide lyases, glycoside hydrolases, a pathogenesis-related protein, a hypersensitive-inducing protein, peptidases, carbohydrate esterase and several hypothetical proteins (**Additional File 9**). The number of predicted effectors in the other five

Diaporthe species ranged from 85 to 123 (**Figure 2A; Additional File 9**). *D. caulivora* shared between 63 to 98 effectors candidates with the other five *Diaporthe* species (**Figure 2C**). Nine effector candidates were considered core effectors since they were present in all *Diaporthe* species, including four CAZymes (pectate lyase, polysaccharide lyase, 1,4-beta-D-glucan cellobiohydrolase and xylanase), a protein CAP22 and four hypothetical proteins. Most of the *D. caulivora* effector candidates were also found in other *Diaporthe* species and other fungi, 11 were *Diaporthe*-specific and four were *D. caulivora*-specific (**Figure 3B, Additional File 6**). All *Diaporthe*-specific and *D. caulivora*-specific effector candidates are hypothetical proteins, which lack a conserved domain. Moreover, of the total secreted *D. caulivora* effector candidates, only 17 were identified in the PHI-base; 8 were related to reduced virulence mutant phenotypes and four were identified as effectors (**Additional File 8**). These virulence factors include several CAZymes, comprising four CAZymes of the core effectors, and a sterigmatocystin biosynthesis peroxidase involved in toxin production [51]. Taken together, our results revealed that the genome of *D. caulivora* has a large array of pathogenicity-related genes, most of which are in common with other *Diaporthe* and fungal pathogens, while others are *Diaporthe*-specific or *D. caulivora*-specific. Further studies are needed to reveal the function of these genus- and species-specific effector candidates.

D. caulivora **genes encoding virulence factors and effector candidates are induced during soybean Infection**

In order to identify *Diaporthe* genes involved in pathogenicity, we performed transcriptional profiling of two early stages of *D. caulivora* infection of soybean plants (8 and 48 hpi) and included *D. caulivora* mycelium grown on PDA medium (**Additional File 10**). Reads mapped uniquely to *D. caulivora* genome, 69,940,418 reads, were considered for further analyses. Biological variability within replicates was analyzed by principal component analysis (PCA). As shown in **Figure 5A**, the first principal component (PC1) accounted for 79,1% of the total variation and separates the two time points (8 and 48 hpi), and the control *D. caulivora* samples. In total, 306 *D. caulivora* genes were differentially expressed in plant tissues (48 vs 8 hpi), 295 genes were upregulated and 11 downregulated (**Figure 5B; Additional File 11**). In order to obtain more information on the infection process, we also compared differential expression between samples at 8 hpi and 48 hpi with

mycelium samples grown on PDA. We identified 2635 additional *D. caulivora* differentially expressed genes (DEGs); 77 and 593 were upregulated, and 595 and 1561 were downregulated at 8 and 48 hpi, respectively (**Figure 5B-C; Additional File 11**). We further focused on the total upregulated DEGs of the three comparisons (806 genes), since they could encode pathogenicity-related proteins involved in *D. caulivora* infection strategies. The functions of these DEGs were significantly enriched in several Molecular Function enriched GO terms, including oxidoreductase activity, hydrolase activity, lyase activity, ion binding and transporter activity (**Figure 6A; Additional File 11**).

We identified a high proportion of DEGs showing homology to genes previously reported to be involved in fungal infection processes. During *D. caulivora* infection, genes encoding CAZymes, proteins involved in detoxification and transport of toxic compounds, proteases and effectors were upregulated (**Additional File 11**). A search against the PHI-base, predicted 119 upregulated *D. caulivora* genes that may be involved in pathogenicity (**Figure 6B, Additional File 12**). Among them, 46 were related to reduced virulence or loss of pathogenicity mutant phenotypes and 15 to effectors, including CAZymes such as lectins, xyloglucan-specific endo-beta-1,4-glucanase, pectin and pectate lyases, as well as proteases, peptidases, among others (**Additional File 12**). Of the total upregulated DEGs, 246 encode proteins that were present in our predicted secretome (**Figure 6B**). Interestingly, 106 (43 %) of these upregulated secreted proteins belong to the core secretome, indicating their contribution to *Diaporthe* pathogenesis (**Additional File 12**). Moreover, of the 104 upregulated *D. caulivora* genes encoding secreted CAZymes (**Figure 6B**), 47 were present in the core secretome (**Additional File 11**), including polygalacturonases, endoglucanases, exoglucanases, pectate lyases, pectin lyases, glycoside hydrolases, xyloglucan-specific endo-beta-1,4-glucanase, mannan endo-1,4-beta-mannosidase, rhamnogalacturonan acetylesterases, among others. The number of upregulated DEGs and expression levels of CAZymes-encoding genes, including PCWDEs, increased at 48 hpi compared to 8 hpi (**Additional File 11**), highlighting the important role played by these enzymes in soybean tissue breakdown and host penetration. In addition, seven genes encoding secreted proteins present only in *D. caulivora* and not in other *Diaporthe* species were upregulated, including a kievitone hydratase and six hypothetical proteins, three of which are *D. caulivora*-specific with no hit in public databases. Interestingly, kievitone

hydratases are involved in detoxification processes of the phenylpropanoid kievitone, which play an important role in legumes defense [52]. Of the total number of *D. caulivora* effectors, 23,3% (31 genes) were upregulated, including those encoding several CAZymes, a cell wall glycoprotein, several small secreted proteins, and hypothetical proteins (**Additional File 11,12**). Interestingly, five of the nine core effectors were upregulated during infection, including a xylanase, a polysaccharide lyase, CAP22, a putative 1,4-beta-D-glucan cellobiohydrolase and a hypothetical protein, indicating that they represent common effector candidates in different *Diaporthe* plant pathogens. Most of these common genes encode proteins involved in cell wall degradation and modification, and CAP22 is expressed in other pathogenic fungi in infection structures such as appresoria [53]. Moreover, one *Diaporthe*-specific (gene_10233.t1) and one *D. caulivora*-specific (gene_06736.t1) effector candidates were upregulated during infection.

Further inspection in transcriptomic data revealed possible strategies used by *D. caulivora*, and probably the other *Diaporthe* species, leading to host infection and tissues colonization. In addition to CAZymes-encoding genes, other upregulated genes belonging to the core secretome include proteins involved in pathogenesis such as peroxidases, SnodProt1, necrosis- and ethylene-inducing peptide 1 (Nep1)-like protein, saponin hydrolase, pathogenesis related protein, CAP22, peptidases, small secreted proteins and lipases. Upon entry into the host, fungal pathogens must resist toxic compounds produced by the plant [54]. Upregulation of a high number of genes encoding proteins involved in oxidoreductase processes during *D. caulivora* infection, like dehydrogenases, oxidases and reductases, is consistent with their important role played during fungal colonization and maintenance of redox status in both organisms [55] (**Additional File 11,12**). Several upregulated *D. caulivora* peroxidase-encoding genes belonging to the core secretome showed homology with genes included in the PHI-base. Like in other plant-pathogen interactions, *D. caulivora* and other *Diaporthe* peroxidases could be involved in lignin breakdown and detoxification of ROS produced by the host. In addition, expression of aldehyde dehydrogenases-encoding genes increased upon *D. caulivora* infection, which could be involved in pathogenicity through scavenging reactive aldehydes, fatty acid radicals, and other alcohol derivatives, as occur in other plant fungal pathogens [56] (**Additional File 12**). Moreover, 24 cytochrome P450 encoding genes were upregulated during *D. caulivora* infection, several of which exhibited functionally characterized homologs in PHI-base (**Additional File 12**). Since some

277 cytochrome P450 are capable of detoxifying phytoalexins [57], and P450 monooxygenases are involved in
278 the synthesis of some fungal toxins [58], they probably are also needed for *D. caulivora* pathogenesis.
279 Interestingly, P450 families expanded significantly in several plant pathogens, including *D. ampelina*
280 indicating their important roles during fungal pathogenesis [30]. Enzymes with oxidative-reductive
281 properties also play relevant functions in degrading antimicrobial compounds such as phytoalexins and
282 polyamines of plant origin [59]. Consistently, *D. caulivora* genes encoding enzymes involved in detoxification
283 of plant defense molecules show high expression levels during early infection stages. Among them, we found
284 several genes encoding putative pisatin demethylases, most of which were related to reduced virulence
285 phenotypes in other pathosystems according to PHI-base (**Additional File 12**). In *Fusarium oxysporum* f. sp.
286 *pisi*, a pisatin demethylase is responsible for detoxifying the pea phytoalexin pisatin [60]. Pisatin
287 demethylases could encode glyceollin demethylases and detoxify soybean phytoalexin glyceollin [59].
288 Furthermore, two genes encoding dienelactone hydrolase (gene_04049.t1, gene_11950), involved in the β-
289 ketoadipate pathway [61], were highly expressed, suggesting that they could be involved in detoxification
290 mechanisms of plant defensive aromatic compounds. Interestingly, a saponin hydrolase present in the core
291 secretome was upregulated during *D. caulivora* infection. These type of enzymes hydrolyze plant saponins
292 that have antifungal activity and serve as potential chemical barriers against pathogens [62]. Thus, they could
293 be part of *Diaporthe* strategies to achieve successful host infection. On the other hand, a Kievitone hydratase
294 encoding gene (gene_03751.t1), which was only present in *D. caulivora* and other fungi and not in other
295 *Diaporthe* species, was upregulated during *D. caulivora* colonization. Kievitone hydratase is an important
296 virulence factor in *Fusarium solani* that catalyzes the conversion of bean kievitone to a less toxic metabolite
297 [48]. Furthermore, a gene encoding the phytotoxin SnodProt1, present in the core secretome, displays
298 increased expression throughout the *D. caulivora* infection process. These type of proteins are involved in
299 plant tissue colonization by several pathogenic fungi and produces ROS and necrosis of the plant tissues [63].
300 We have observed that *D. caulivora* infection induces ROS production in soybean stems (Mena, unpublished
301 observations), and *D. aspalathi* elicitors activate the formation of nitric oxide (NO) in plant tissues that
302 triggers the biosynthesis of antimicrobial flavonoids in soybean [64]. These findings, suggest that ROS and
303 NO production play a role in plant-*Diaporthe* interactions.

Fungal toxins and necrosis inducing factors are produced to kill plant cells enabling nutrient uptake and mycelium growth. During *D. caulivora* infection, genes encoding secreted NEP (gene_04209.t1, gene_05426.t1 and gene_11576.t1) and a hypersensitive response-inducing protein (gene_18357.t1) were upregulated. Two of these NEP proteins belong to the core secretome, indicating their contribution to *Diaporthe* pathogenesis. Expression levels of genes involved in mycotoxin biosynthesis (gene_13234.t1, gene_10937.t1, gene_00256.t1, gene_05355.t1 and gene_15459.t1), and 11 genes encoding polyketide synthases (PKS) involved in toxin production in other fungi, increased during soybean infection (**Additional File 12**). Upregulation of this high number of PKS-encoding genes underline their involvement in *D. caulivora* pathogenesis, which is consistent with PHI-base hits with reduced virulence mutant phenotypes for PKS (gene_13009.t1, gene_01287.t1, gene_16446.t1) (**Additional File 12**), and previously reported reduced virulence of *D. helianthi* PKS1 mutant [27]. During sunflower infection, *D. helianthi* produces the polyketidic phytotoxin phomozin and related toxins, and purified phomozin causes disease symptoms [27]. Interestingly, the possible involvement of a phytotoxin produced by *D. caulivora* during SCC development has been previously reported [65].

Enzymes such as subtilases and alkaline proteinases have been shown to degrade plant defense proteins [66]. Increased expression of genes encoding three subtilisin-like proteinases (gene_11104.t1, gene_05325, gene_14820), an aspartic proteinase (gene_08152), and several other proteases in *D. caulivora*-infected tissues suggests their possible involvement in degradation of plant defense proteins. Consistently, disruption of genes with significant homology to gene_11104.t1 and gene_08152.t1, as well as other genes encoding proteases (gene_15501.t1, gene_15174.t1 and gene_06260.t1) showed reduced virulence in other plant pathogens (**Additional File 12**). The role of proteases and peptidases in *Diaporthe* pathogenesis is further supported by their presence in the core secretome.

Other upregulated genes encoding virulence factors include several transporters such as major facilitator superfamily (MSF) and ABC transporters, that in plant pathogenic fungi are responsible not only for export of compounds involved in pathogenesis, but also for excretion of plant-derived antimicrobial compounds [67]. In total, 6 MSF and four ABC transporters were upregulated during *D. caulivora* infection and showed

significant homology to other transporters involved in virulence mechanisms in other plant pathogens (**Additional File 11, 12**).

Taken together, our results suggest that early infection processes of soybean stems by *D. caulivora* depends on plant cell wall degradation and modification, detoxification of toxic compounds, transporter activities and toxin production. In addition, increased expression of genes encoding putative effector proteins during host colonization, some of which are species-specific, indicate that *D. caulivora* infection strategy also relies on plant defense evasion. Interestingly, several of these upregulated genes encoded proteins that are part of the core secretome and represent common virulence components among *Diaporthe* species.

Conclusions

Our findings give novel and relevant insights into the molecular players involved in pathogenicity of *D. caulivora* towards soybean plants, several of which are in common with other *Diaporthe* pathogens with different host specificity, while others are species-specific. Future studies towards understanding the role of effector candidates during the infection process of important pathogens among *Diaporthe* genus could reveal novel effector functions and plant targets that underpin *Diaporthe* pathogenic lifestyle. Our findings improve our understanding of *D. caulivora* pathogenicity mechanism involved in SCC development and allow genomic and transcriptomic comparisons among the most important pathogens belonging to the *Diaporthe* genus. Finally, the knowledge generated in this study provides a foundation for developing effective disease management strategies for SSC.

Methods

All methods were performed in accordance with the relevant guidelines and regulations.

D. caulivora inoculation

The virulent strain D57, isolated from a stem canker lesion of a soybean plant grown in Uruguay [13], was growth in potato dextrose agar (PDA; Difco, Detroit, USA) at 24°C in 12 h light/12 h darkness photoperiod. Plugs of a 5-day-old culture were used for plant inoculation. SSC-susceptible soybean (*Glycine max*) plants

cultivar Williams (PI548631), obtained from USDA ARS Soybean germplasm collection (seed source 13U-9280), were used for all assays. Soybean seeds were planted in 12-cm x 12-cm pots filled with a mix of soil and vermiculite at a rate of 3:1. Plants were grown at 24°C under a 16 h light/8 h dark lighting regime. Soybean inoculation was performed using the stem wounding method described by Mena *et al*. [13], and 3-week-old (V2 stage) plants were inoculated with mycelium containing plugs.

Genomic DNA extraction, sequencing and de novo *D. caulivora* genome assembly

Agar plugs (5 mm in diameter) from the growing edge of 8-day-old cultures grown on PDA were transferred to new culture media and incubated at 24°C under 12h light and dark photoperiod. After 7 days, when the fungal growth completed the Petri dish, the mycelia were collected from three Petri dishes and frozen with liquid nitrogen. Approximately 150 mg of mycelium was ground with liquid nitrogen. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and quantified with Nanodrop. PacBio library preparation and sequencing were done in the Integrative Genomics Core Service, Beckman Research Institute, City of Hope (Monrovia, CA, USA). Briefly, a library for single-molecule real-time (SMRT) sequencing was constructed with an insert size of 15 kb using the SMRTbell™ Template Prep kit (Pacific Biosciences of CA, USA). The size of inserts was determined using the BluePippin device. Finally, the whole genome of *D. caulivora* was sequenced using the PacBio Sequel platform. Subreads were obtained using the SMRT Analysis RS. Flye v.2.7 [68] was used to assemble the data applying standard parameters and an estimated genome size of 50 Mb. The polishing and correction steps are included in the assembly pipelines. The genome graph structure was visualized with Bandage [69] to survey contiguity and ambiguities. Assembly statistics were obtained with QUAST v5.0.2 [70].

Gene prediction and functional annotation

Gene prediction was performed using the FunGap pipeline [71]. Briefly, the gene prediction was performed using three publicly available programs, Augustus [72], Braker 1 [73] and Maker [74] housing into the pipeline. Transcriptomic data of *D. caulivora* strain D57 obtained with the RNASeq protocol and adapter and quality trimmed using Trimmomatic v0.39 [75] was used to aid the gene model prediction. The parameters

using to run FunGAP were: –sister_proteome: Fusarium, – augustus_species fusarium_graminearum and transcript reads as –trans_read: paired-end. *Fusarium graminearum* was used as the reference species due to the relatively close phylogenetic relationship to *Diaporthe* among the genome sequences available in GenBank (Li *et al* 2017). Single-copy fungal orthologs (fungi_odb10.2019-11-20 gene set) from Benchmarking Universal Single-Copy Orthologs (BUSCO v4) [76] were used to assess the completeness of the genome annotation.

Functional annotation was completed with the Blast2GO [44], through Omicbox software (https://www.biobam.com/omicsbox). Gene models where compared with several databases (NCBI nonredundant protein database, GO, and InterpoScan) with BlastP finding single hit at an e-value threshold of 1E-20 using taxIds for fungi [77]. InterproScan analysis was used to identify domains in the *D. caulivora* genome [78]. Classification into gene ontology (GO) categories was performed with Blast2GO software using *Fusarium graminearum* as a reference since protein domain information for *D. caulivora* was not available [79].

Comparative genome analyses

The genomes of *D. capsici* strain GY-Z16 (Gene Bank accession number: WNXA00000000); *D. citri* strain NFHF-8-4 (Gene Bank accession number: JACTAD000000000); *D. destruens* strain CRI305_2 (Gene Bank accession number: JACAAM000000000); *D.longicolla* strain TWH P74 (Gene Bank accession number: JUJX00000000); *D. phragmatis* strain NJD1 (Gene Bank accession number: JACDXY000000000) were downloaded from GenBank database of the NCBI website ((https://www.ncbi.nlm.nih.gov/nuccore).

Gene prediction for each *Diaporthe* assemblies downloaded was performed using AUGUSTUS web Server (http://bioinf.uni-greifswald.de/webaugustus/), with *D. caulivora* gene models from this work as a training set with default parameters (UTR prediction: false; report genes on: both strands; alternative transcripts: medium; allowed gene structure: predict any number of (possibly partial) genes.). The average nucleotide identity (ANI) of all Diaporthe species was calculated using PYANI v0.3. 0-alpha with MUMer to align the input sequences (ANIm). (https://github.com/widdowquinn/pyani). The orthologous analysis was

conducted using OrthoFinder v2.5.4 [80] with all-versus-all BLAST strategy to define the orthogroups among the six *Diaporthe* species. Phylogenetic species **rooted** tree **was inferred by multiple sequence alignment and maximum likelihood (options: "-S blast -M** msa") for all orthogroups based on Species Tree Inference from All Genes method (STAG).

Multiple software tools were jointly used to predict secreted proteins of the six *Diaporthe* species. SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/) [81], WoLF PSORT for Fungi (http://www.genscript.com/psort/wolf_psort.html) [82], and Phobius (http://phobius.sbc.su.se/) [83], were employed to identify signal peptide signatures. Default parameters were used for all programs. For WoLF PSORT, proteins were considered if their extracellular score was ≥ 17 [84]. Proteins predicted to be signal-peptide positive by all three programs were taken and two additional filtering steps were performed to remove possible membrane proteins using TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) [85], removed proteins with expected number of amino acids intransmembrane < 18, and possible endoplasmic reticulum (ER) targeting proteins, predicted by using PS-Scan (http://prosite.expasy.org/scanprosite/) [86] with Prosite accession PS00014. The remaining proteins were considered as secreted proteins and used in subsequent analyses. Additionally, EffectorP V3.0 was used to predict fungal effectors of all *Diaporthe* species [87-89].

The carbohydrate-active enzymes (CAZymes) were identified with dbCAN 5.0, which searches CAZy family-specific HMMs with HMMER3, and NCBI's conserved domain database CDD [90]. Putative polyketide synthases (PKS) genes were identified using InterProScan and identification of conserved domain as indicated in [27]. To identify proteins involved in pathogenicity, the predicted secretome was used as a query for BlastP (e-value 1E-05) search against the pathogen-host interaction database (PHI-base v4.10) that catalogues experimentally verified pathogenicity, virulence and effector genes from fungal, oomycete and bacterial pathogens [47].

RNA Extraction, RNA Sequencing and Data Processing

For transcriptomic analysis, samples were taken at 8 h post inoculation (hpi) and 48 hpi in soybean plants and *D. caulivora* mycelium grown on PDA plates for seven days was used as a control. Each treatment consisted of three pots with three plants each at each infection time point, and three plates of mycelium grown on PDA plates. Soybean tissues (stem section of 1.5 cm including the wounded area) and *D. caulivora* mycelium were harvested for RNA extraction, immediately frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted and purified from 100 mg soybean stems with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and Invitrogen PureLink RNA Extraction Mini kit (Invitrogen, USA), including an on column digestion with RNase-Free DNase I, according to the manufacturer's instructions. Quality of the isolated RNA was checked by running samples on 1.2% formaldehyde agarose gel. RNA concentration was measured using a NanoDrop 2000c (Thermo Scientific, Wilmington, USA). RNA quality control, library preparation, and sequencing were performed at Macrogen Inc. (Seoul, Korea). Three biological replicates are included by treatment. Libraries for each biological replicate were prepared for paired-end sequencing by TruSeq Stranded Total RNA LT Sample Prep Kit (Plant) with 1 μg input RNA, following the TruSeq Stranded Total RNA Sample Prep Guide, Part # 15,031,048 Rev. E. Sequencing was performed on Illumina platform (Illumina, CA, USA) by Macrogen Inc. (Seoul, Korea) to generate paired-end 101 bp reads, obtaining 41.6 to 65.2 M reads per sample with Q20 > 98% and Q30 > 95%. RNA-seq processing steps were done through Galaxy platform (https://usegalaxy.org/). Raw reads quality were subjected to a quality control check using FastQC software ver. 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequences were trimmed, and the adapters removed using Trimmomatic Version 0.38.0 software [75]. Additionally, to the default options, the following parameters were adjusted: adapter sequence TruSeq3 (paired-ended (PE), for MiSeq and HiSeq), always keep both reads of PE, and SLIDINGWINDOW: 4:15 HEADCROP: 13 MINLEN:50.Trimmed reads were mapped to the assembly of the *D. caulivora* genome obtained before using Hisat2 software [91]. The BAM files were obtained with Samtools View software v. 1.9 and then sorted by name with Samtools Sort software v. 2.0.3 [92], for further analysis.

Reads were counted using FeatureCounts software v. 1.6.4 [93]. Additionally to default options, parameters were adjusted for: count fragments instead of reads, allow read to map to multiple features, and use reference sequence file obtained before for *D. caulivora* annotation. Cluster analysis of replicates from each

time point and control samples were performed by Principal Component Analysis (PCA) using pcaExplorer 2.16.0 software [94]. Differential expression analyses were performed using EdgeR software ver. 3.24.1 [95], with p-value adjusted method of Benjamini and Hochberg adjusted threshold 0.05 [96], and Minimum log2 Fold Change 2. Counts were normalized to counts per million (cpm) with the TMM method and low expressed genes filtered for count values ≥ 3 in all samples. In this study, a false discovery rate (FDR) ≤ 0.05 was used to determine significant differentially expressed genes (DEGs) between *D. caulivora* grown on soybean stem and *D. caulivora* grown on PDA (control), and expression values were represented by log2 ratio. Venn diagram drawing was performed with Venny [97].

Abbreviations

CAZymes: carbohydrate-active enzymes

DEG: Differentially expressed gene

GO: Gene Ontology

PCA: Principal Component Analysis

PDA: Potato dextrose agar

PCWDEs: Plant cell wall degrading enzymes SSC: soybean stem canker

PHI-base: Pathogen–Host Interaction base

ROS: Reactive Oxygen Species

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The nuclear assembly data of *D. caulivora* (D57) was deposited into NCBI's Genome database (https://www.ncbi.nlm.nih.gov/genome/) under the BioProject ID: PRJNA717308. All raw RNA-Seq read data

were deposited at NCBI Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/) under the BioProject ID PRJNA717275. The data are under embargo until publication.

Competing interests

The authors declare that they have no competing interests.

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Author´s contributions

EM performed the DNA and RNA extraction, aligned and mapped the reads obtained by RNAseq to the *D. caulivora* genome, analyzed the data and helped to write the manuscript. SG assembled and annotated the *D. caulivora* genome, performed gene identification and comparative genomic analysis, analyzed the data and helped to write the manuscript. SS interpreted the data and contributed to discussions. MM interpreted the data, contributed to discussions and helped to write the manuscript. IPDL supervised the study, interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript.

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Figure legends.

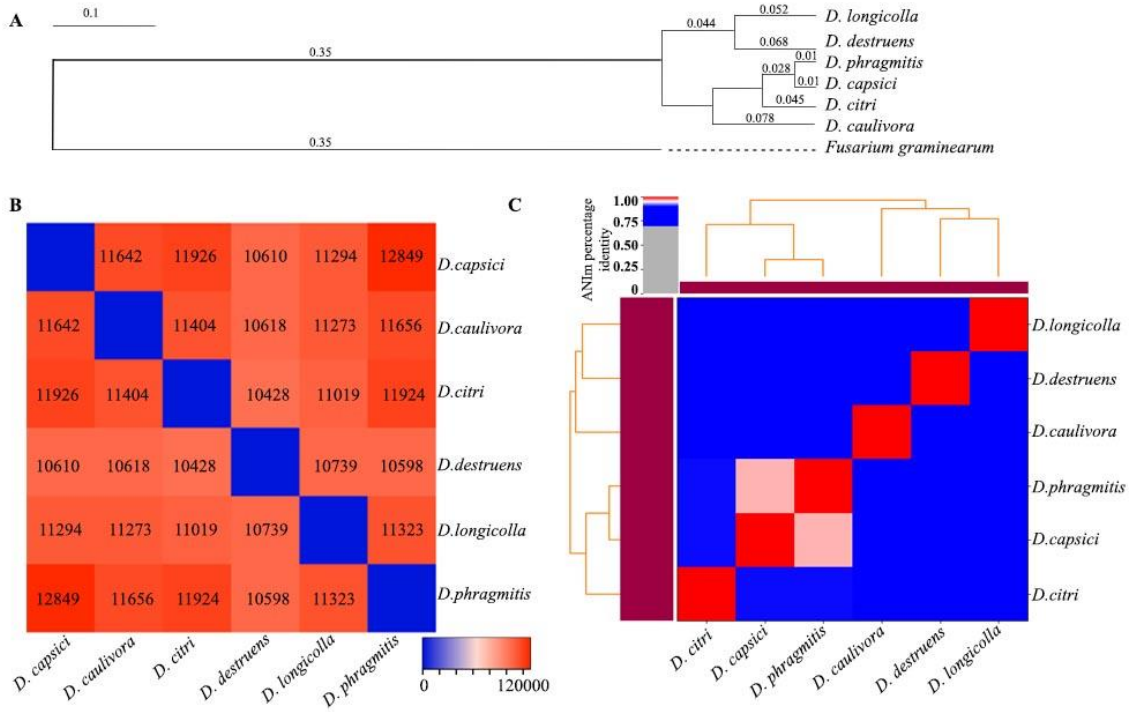
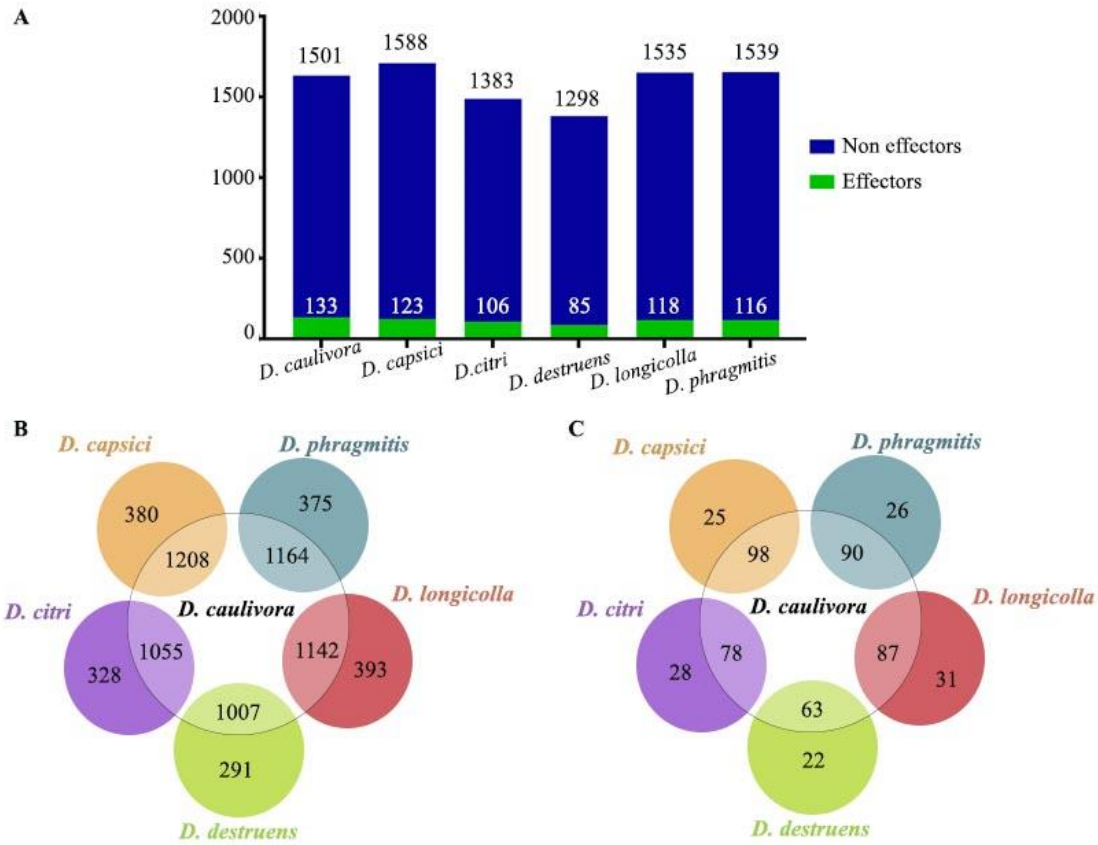


Figure 1: Orthologs and phylogenomic relationships of *D. caulivora* and other pathogenic *Diaporthe* *species*. (A) The species tree based on the orthologous groups was inferred by STAG and rooted by STRIDE. *Fusarium graminearum* was used as outgroup. **(B)** Heatmap showing the number of genes of each fungal species included in the orthogroups obtained by OrthoFinder. The scale represents the number of genes in orthogroups. **(C)** Heatmap of identity percentage amongs *Diaporthe* species based on the average nucleotide identity (ANI) analisys performed using PYANI v0.3. 0-alpha with MUMer to align the input sequences (ANIm). Scale in **(A)** represents divergence and substitutions per site.



754 **Figure 2: Number of predicted secreted proteins and effector candidates in *D. caulivora* and five other**

755 ***Diaporthe* species. (A)** Distribution of effectors in the different secretomes. Number of secreted proteins **(B)**

756 and effector candidates **(C)** shared between *D. caulivora* and other *Diaporthe* species.

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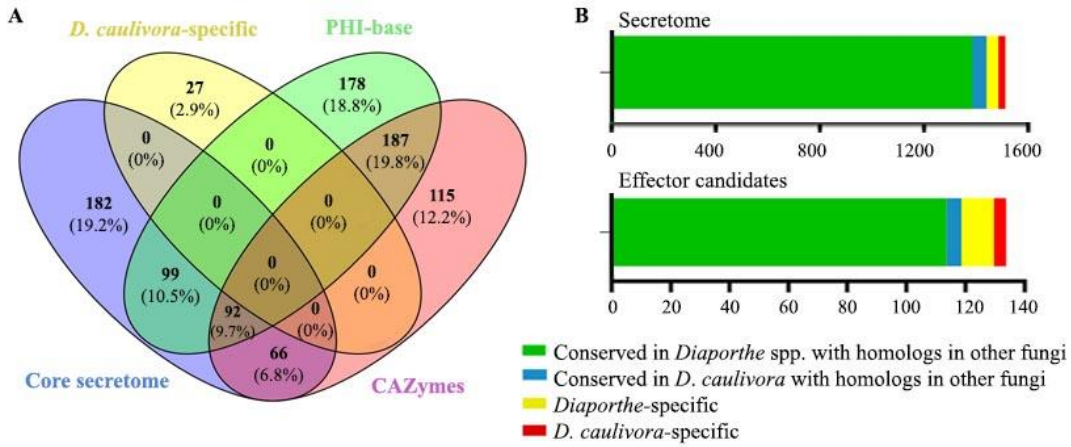
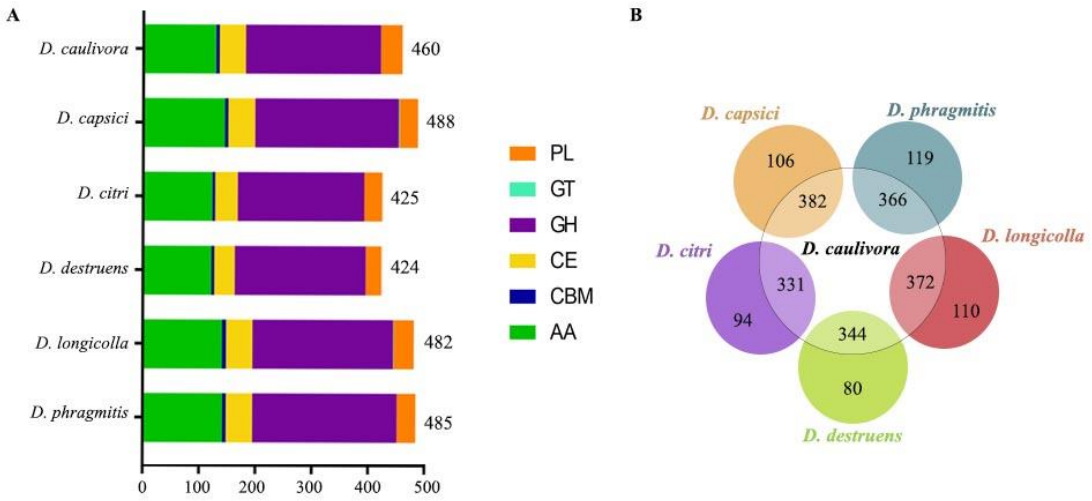


758 **Figure 3: Shared and *D. cualivora*-specific secreted proteins and candidate effectors. (A)** Venn diagram

759 showing the overlap the core secretome, *D. caulivora*-specific secreted proteins, *D. caulivora* secreted

760 CAZymes and PHI-base. **(B)** Conservation patterns of secreted proteins and effector candidates from D.

761 caulivora indicating the number of conserved homologs in other *Diaporthe* species and fungi.



762 **Figure 4: Comparative distribution of secreted CAZymes in different *Diaporthe* species. (A).** Distribution
763 according to classes of CAZyme module. PL, polysaccharide lyases; GT, glycosyl transferases; GH, glycoside
764 hydrolases; CE, carbohydrate esterases; CBM, carbohydrate-binding module and AA, auxiliary activity. **(B)**
765 Number of CAZymes shared between *D. caulivora* and other *Diaporthe* species.

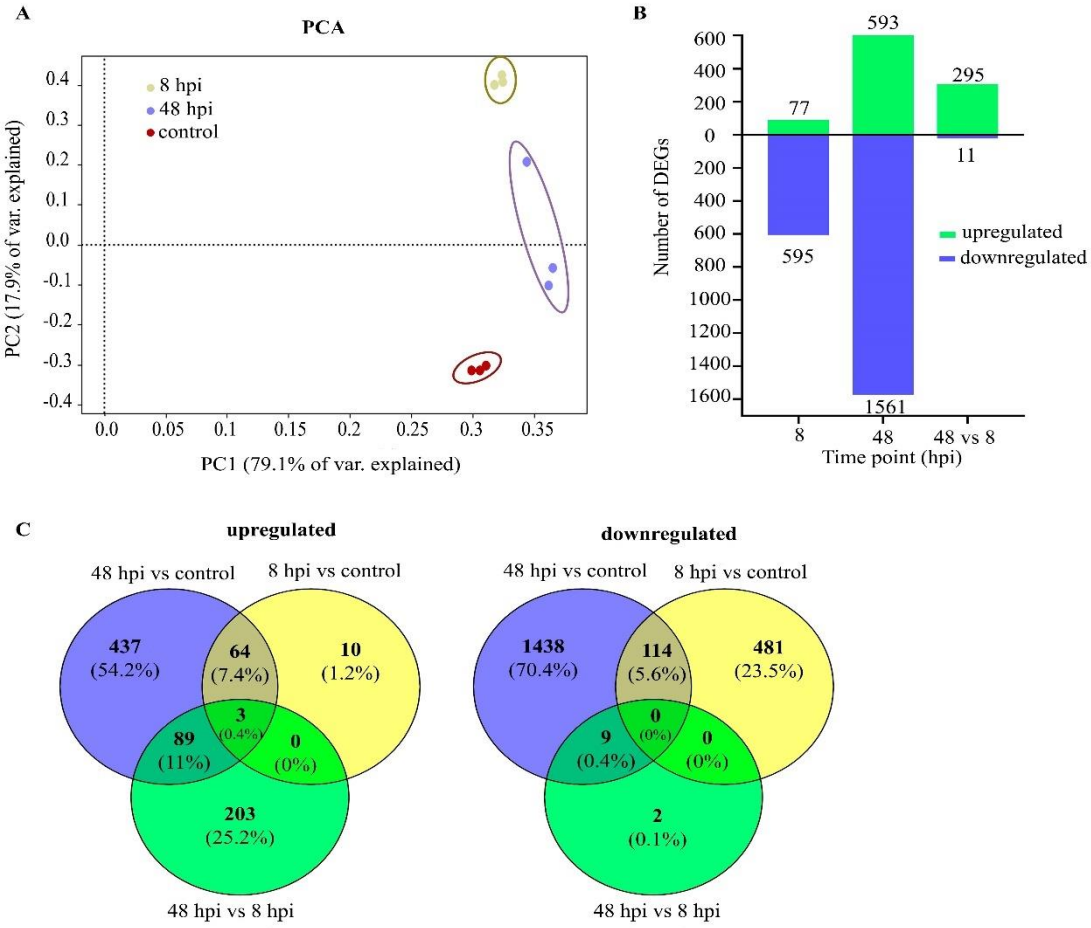


Figure 5: Differentially expressed *D. caulivora* genes during soybean infection. (A) Principal component analysis (PCA) of the transcriptomic data from RNA-seq. Colored dots denote each biological replicate. **(B)** Number of differentially expressed genes (DEGs). **(C)** Venn diagrams from *D. caulivora* comparisons at 8 hpi vs control, 48 hpi vs control and 48 vs. 8 hpi. Log2 FC ≥ 2.0 or ≤ - 2.0 and false discovery rate (FDR) ≤ 0.05 were considered for DEGs identification. In Venn diagrams, the overlap of expressed fungal genes can be observed.

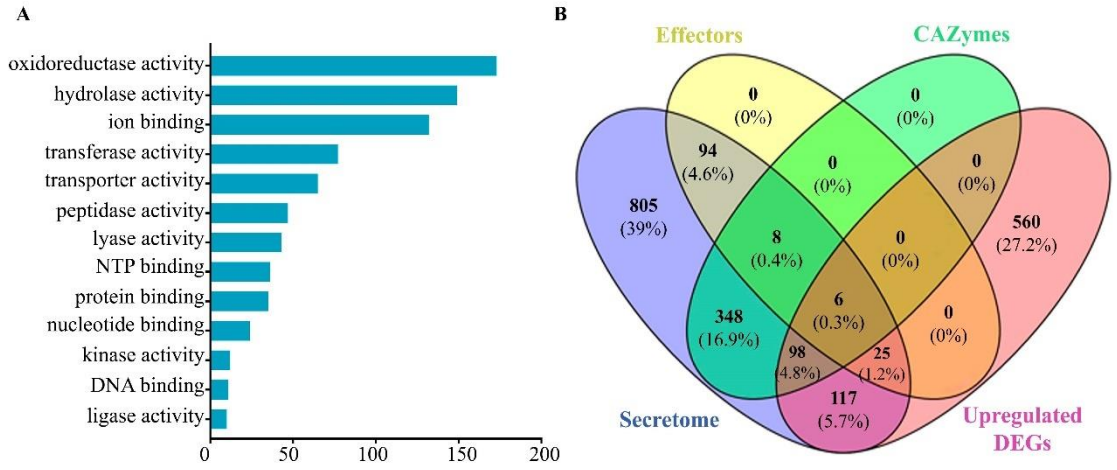
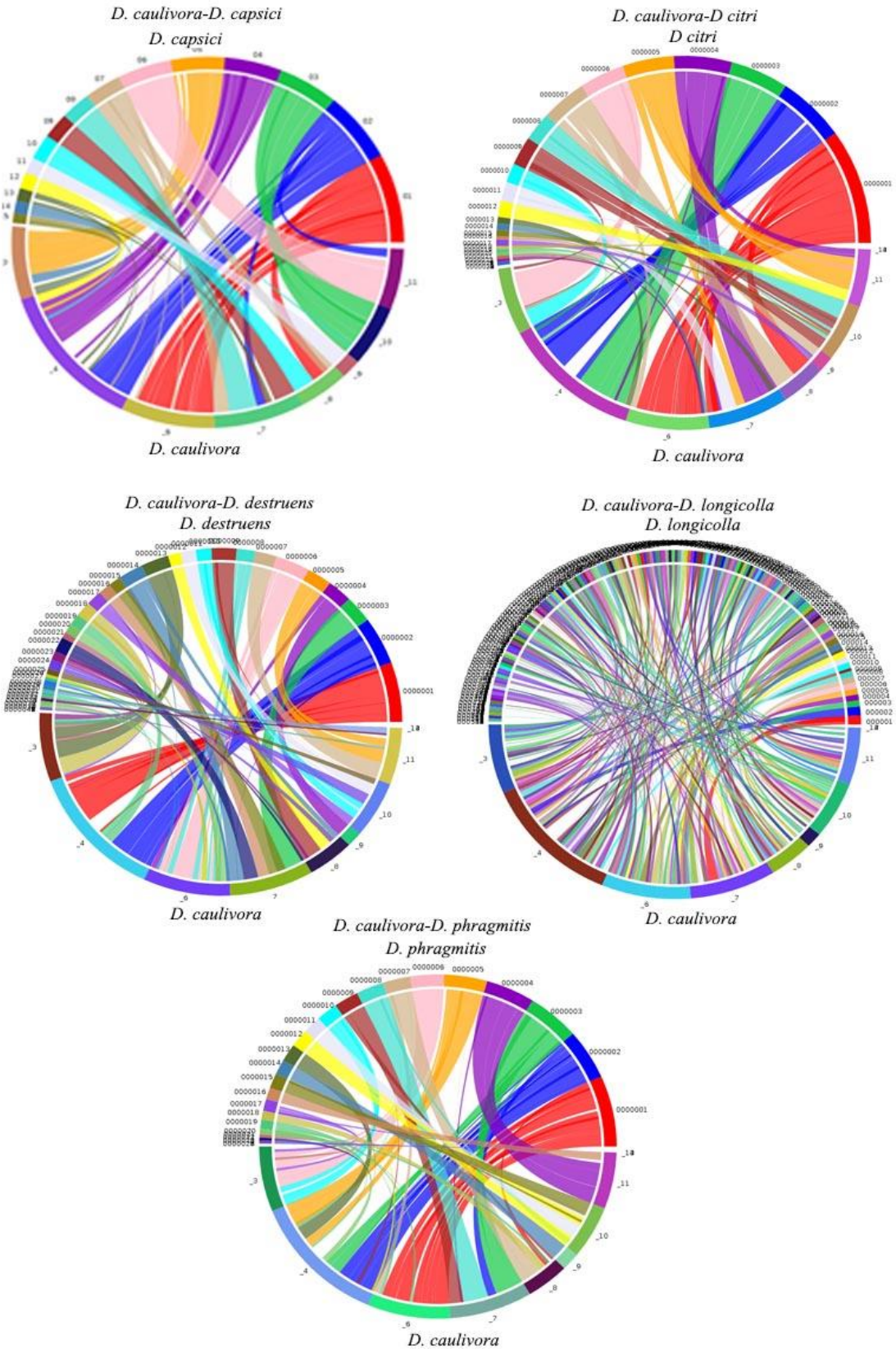


Figure 6: (A) Enriched Gene Ontology (GO) terms distribution for molecular function (MF) of upregulated differentially expressed genes (DEGs) in *D. caulivora* during soybean infection. **(B)** Venn diagram showing the overlap of upregulated DEGs and different gene categories relevant to *D. caulivora* pathogenicity.

Additional Files

Additional File 1: General features of *Diaporthe* genomes.

Additional File 2: Characteristics of selected *Diaporthe* species.



779 **Additional File 3: Synteny analysis between *D. caulivora* and other *Diaporthe* species.** The synteny circle

780 plots show the large synthenic blocks between *D. caulivora* and the other five *Diaporthe* species obtained

781 using SyMAP.

782 **Additional File 4: Gene prediction and annotation of *D. caulivora*.**

Additional File 5: *Diaporthe* genes encoding predicted secreted proteins shared with one or more *Diaporthe* species or present only in one species.

Additional File 6: Predicted *D. caulivora* secreted proteins and effector candidates absent in the other five *Diaporthe* genome. A NCBI search was performed showing hit with other *Diaporthe* and fungal species.

Additional File 7: Predicted CAZymes in the different *Diaporthe* species. CAZymes shared with one or more *Diaporthe* species or present only in one species are indicated.

Additional File 8: Summary of *D. caulivora* genes encoding predicted secreted proteins and effector candidates with functionally characterized homologues in Pathogen-Host Interaction database (PHI-base).

Additional File 9: Effectors candidates in *Diaporthe* secretomes shared with one or more *Diaporthe* species or present only in one species.

Additional File 10: Summary of mapped reads of *D. caulivora* in the RNA-Seq libraries. 1-3 indicate the three biological replicates during soybean infection at the indicated time points and control PDA grown mycelium.

Additional File 11: List of *D. caulivora* differentially expressed genes (DEGs) during soybean infection at 8 and 48 hpi.

Additional File 12: List of pathogenicity related *D. caulivora* differentially expressed genes (DEGs) present in different categories: secretome, CAZymes, effectors, and their functionally characterized homologs in PHI-base.

Capítulo III

Respuestas de defensa basal y tempranas son la base de la resistencia de plantas de soja frente al patógeno fúngico *Diaporthe caulivora*

En esta última parte del trabajo, se comparó la infección de *D. caulivora* y el avance de la enfermedad entre dos genotipos de soja Williams (susceptible) y Genesis 5601 (resistente). Genesis 5601 fue obtenido a partir del programa de mejoramiento genético del Instituto Nacional de Investigaciones Agropecuarias (INIA), mientras que Williams fue obtenido del Departamento de Agricultura de los Estados Unidos (USDA). La inoculación y el seguimiento de la infección se realizaron igual que en el capitulo I y se midieron el largo de las lesiones, el índice de severidad, el área bajo la curva del progreso de la enfermedad y la biomasa del patógeno en ambos genotipos. Los resultados obtenidos mostraron un mayor grado de infección en las plantas de Williams, siendo Genesis 5601 más resistente como habíamos observado en estudios preliminares. Para comprender las bases moleculares de la interacción, se hizo un abordaje del perfil transcriptómico vegetal en los primeros estadíos de infección con *D. caulivora* (0, 8 y 48 hpi). En este caso se analizó en paralelo la respuesta diferencial de los dos genotipos contrastantes; Williams y Genesis 5601. A tiempo 0 sin la presencia del patógeno se expresaron diferencialmente 164 genes, 73 de ellos estaban inducidos en Genesis 5601 respecto a Williams entre los que destacan receptores y genes relacionados con la defensa. Este resultado muestra la diferencia basal de Genesis 5601 que podría explicar, en parte, el nivel de resistencia observado.

En respuesta a la infección con *D. caulivora*, en total se expresaron diferencialmente 1855 y 2322 genes en Williams y Genesis 5601, respectivamente. La inducción de genes en Genesis 5601 fue mayor y se evidenció una respuesta más rápida con respecto al genotipo susceptible Williams a las 8 hpi. El patrón de expresión de los genes regulados positivamente y el análisis de enriquecimiento mostraron que las vías de señalización hormonal y la ruta de los fenilpropanoides tiene un rol fundamental en la respuesta de defensa frente a *D. caulivora*. Además, observamos la inducción de genes que codifican receptores, proteínas quinasas, proteínas relacionas con la patogénesis, transportadores y factores de transcripción. El análisis del transcriptoma de plantas inoculadas con *D. caulivora* respalda los resultados obtenidos en el capitulo I donde se indujeron genes de defensa. Los genes que codifican para PR-1, PR-4, PR-10, peroxidasa, PAL y CHS fueron inducidos en ambos genotipos de soja después de la inoculación con *D. caulivora*. Se indujeron un mayor número de estos genes en Genesis 5601 que en Williams.

De acuerdo con nuestros resultados las hormonas auxinas y etileno, que representan el 32 % y 33 % de los genes de las vías hormonales, están involucradas en la resistencia/susceptibilidad de las plantas de soja frente a *D. caulivora.* Le siguen en

relevancia las vías del ácido abscísico y el ácido salicílico. En todos lo casos la mayor cantidad de genes con altos niveles de expresión se encontró en el genotipo resistente Genesis 5601. El ácido salicílico, el ácido jasmónico y el etileno estan relacionado con los mecanismos de defensa frente a patógenos (Pandey and Senthil-Kumar, 2017). La inducción de enzimas de la síntesis de etileno está relacionada con la resistencia de plantas de soja a patógenos como *Fusarium virguliforme* (Abdelsamad *et al*., 2019).

La inducción de genes vegetales de la ruta de los fenilpropanoides evidencia la activación de la síntesis de diversos metabolitos secundarios como flavonoides, isoflavonoides, lignina y otros compuestos con actividad antimicrobiana (Vogt, 2010; Piasecka *et al*., 2015). La gran cantidad de genes vegetales que codifican para peroxidasas en las plantas inoculadas con *D. caulivora* respalda la teoría de la síntesis de lignina (Wang *et al*. 2013). La ruta de los fenilpropanoides ha sido inducida en plantas de soja frente a otros patógenos como *Pseudomonas syringae, Rhizoctonia solani* y *Sclerotinia sclerotiorum* (Zabala *et al*., 2006; Chen *et al*., 2010; Ranjan *et al*., 2019).

Por otro lado, el reconocimiento de patógenos y la señalización son los primeros pasos en la activación de los mecanismos de defensa de la planta frente a patógenos. Nuestros resultados reflejan la activación temprana de varios receptores de reconocimiento de patógenos y proteínas quinasas en el genotipo resistente Genesis 5601. Receptores tipo proteínas kinasas (RLK), receptores tipo proteínas (RLP), receptores ricos en cisteína (CRKs) han sido referidos por otros autores por sus altos niveles de expresión en plantas de soja tratatadas con patógenos o con PAMPs (McCabe *et al*., 2018; Delgado-Cerrone *et al*., 2018). Los genes de las cascadas de proteínas quinasas se activan también en plantas de soja frente a *Fusarium oxysporum* (Lanubile *et al*. 2015) y *Heterodera glycines* (McNeece *et al*., 2019).

La inducción de genes R y proteínas relacionadas con la patogénesis (PR) a las 0 hpi en Genesis 5601 y a 8 y 48 hpi en este genotipo y a las 48 hpi de Williams en plantas inoculadas con *D. caulivora* evidencia la respuesta diferencial de los genotipos. En correspondencia con los resultados obtenidos, se han identificado genes *Rpg*, *Rbs, Rdm*, *Rps, Rpp* y *Rhg* en plantas de soja que confieren resistencia frente a *Pseudomonas glycinea, C. gregata, D. aspalathi, P. sojae, P. pachyrhizi* y *H. glycines* respectivamente (Ashfield *et al*., 2012; McCabe *et al*., 2018; Chiesa *et al*., 2009; Pioli *et al*., 2003; Zhong *et al*., 2018; Kashiwa *et al*., 2020; Jiao *et al*., 2015). Las proteínas PR se acumulan frente a la invasión de patógenos o durante el estrés abiótico y tienen un rol importante en los mecanismos de defensa (van Loon *et al*. 2006; Jain and Khurana, 2018). Al igual que en nuestros resultados varias PR se encuentran inducidas en plantas de soja frente a patógenos (Lanubile *et al*. 2015; Zeng *et al*., 2017; Mena *et al*., 2020).

Por último, nuestro análisis reflejó sobreexpresión de los factores de transcripción principalmente WRKYs y de etileno (ERFs). Varios WRKYs han sido identificados en plantas de soja para conferir resistencia a patógenos como *P. pachyrhizi, Fusarium oxysporum, C. gregata, Peronospora manshurica,* y *H. glycines* (Bencke-Malato *et al*., 2014; Lanubile *et al*., 2015; McCabe *et al*., 2018; Dong *et al*., 2018; Jiang *et al*., 2020) y la sobreexpresión de ERFs en plantas transgénicas aumenta la resistencia frente a diferentes tipos de patógenos (Huang *et al*., 2004).

La respuesta diferencial de los perfiles transcriptionales de las plantas de soja Williams y Genesis 5601 frente a la infección con *D. caulivora* refuerza los resultados obtenidos en la comparación del avance de la enfermedad y explica el grado de susceptibilidad/resistencia de los genotipos. De acuerdo al análisis de la literatura, hasta el momento, es el primer estudio de transcriptomas en plantas de soja inoculadas con *D. caulivora* donde además se comparan dos genotipos de plantas con respuesta diferencial a la enfermedad del cancro del tallo. Los conocimientos generados sirven de base para el estudio de genes o familias de genes que confieren resistencia a *D. caulivora* y los mismos podrían ser considerados en los programas de mejoramiento genético para la obtención o generación de líneas de soja resistentes a la enfermedad.

Basal and early defense responses as the basis of soybean resistance against the fungal pathogen *Diaporthe caulivora*

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Abstract

Diaporthe caulivora causes soybean stem canker (SSC), one of the most important disease that leads to losses in crop production. Strategies used to control the disease are not sufficient or satisfactory and the development of resistant varieties is postulated as a viable and promising strategy. The present study aimed to give insights into the molecular mechanisms involved in the resistant response of soybean against *D. caulivora* using two contrasting soybean genotypes. *D. caulivora* causes disease in both cultivars, Genesis 5601 (resistant) and Williams (susceptible), although greater lesion length, severity of the disease, and biomass of the pathogen was observed in the susceptible cultivar Williams compared to Genesis 5601. Differential gene expression was compared by RNA-seq in both cultivars in *D. caulivora*-inoculated and control tissues at 0, 8 and 48 hours post inoculation (hpi). In total, 1855 and 2322 genes were differentially expressed in Williams and Genesis 5601, respectively. At time zero, 164 genes were differentially expressed between both cultivars. In Genesis 5601, 73 genes were upregulated, including membrane receptors and genes related to plant defense. A faster defense response was observed in Genesis 5601 with 1028 upregulated genes at 8 hpi and only 434 genes in Williams. Transcription profiles and gene ontology enrichment analysis of upregulated genes revealed early perception of the pathogen together with defense activation through defense hormones, biosynthesis of phenylpropanoids, and induction of PR genes and overexpression of transcription factors. The faster response of Genesis 5601 together with basal differences compared to the susceptible Williams cultivar explain the degree of resistance / susceptibility observed in our work. These findings present useful knowledge for the development of breeding programs to manage SSC.

Keywords: Transcriptome, soybean, soybean stem canker, *Diaporthe caulivora*, differential expression genes, defense genes

Introduction

Soybean (*Glycine max* L.) is a major crop worldwide that provides abundant amount of protein and oil for human and animal consumption (Dong et al., 2018). Soybean production is affected by biotic and abiotic stress, including fungal pathogens,

oomycetes and adverse environmental conditions such as drought, nutrient deficiency, salt and cold (He at al., 2020). One important disease caused by fungal pathogens is soybean stem canker (SSC) caused by *Diaporthe* species. *D. caulivora* is one of the principal agents causing SSC in Uruguay (Stewart 2015, Mena et al., 2020). Disease symptoms appear on the stem as 1-2 mm dots that expand as elongated brown lesions (Pioli et al., 2003). SSC control is based in integrating management practices, and some fungicides can be applied to reduce SSC infection. The most effective way to control SSC is to develop and use resistant cultivars. Five *Rdm* genes confer resistance against *D. aspalathi*, which is another species that causes SSC, although these resistant genes are not effective against *D. caulivora* (Pioli et al., 2003). Recently, a *Rdc1* gene associated with chromosome 13 of *G. max* was identified as a resistance source for *D. caulivora* (Peruzzo et al., 2019). However, until present there are no commercial *G. max* resistant cultivars to the disease.

Plants are in constant interaction with pathogenic microorganisms, including fungi, bacteria, viruses and oomycetes. The plant recognizes the pathogen and triggers cellular and molecular changes associated with defense responses such as signaling, transcriptional activation, synthesis of defense molecules and their transport to specific sites in the plant (Spoel and Dong, 2012). Two types of defense response have been described: PAMP Triggered Immunity (PTI), that recognizing a broad range of molecular patterns associated with pathogens (PAMPs) or damage (DAMPs) through membrane receptors and Effector Triggered Immunity (ETI) in which pathogen effectors are recognized by specific R genes that encode intracellular nucleotide binding site and NBS-LRR domains (Jones and Dang 2006; Macho and Zipfel, 2014). PTI and ETI eventually converge into many similar downstream responses, albeit with distinct amplitudes and dynamics (Yuan et al., 2021). ETI have greater intensity compared to PTI and generally leads to cell death programs that constitute the so-called hypersensitive response (HR), a type of programmed cell death that restricts the pathogen to the site of infection (Macho and Zipfel, 2014). Both PTI and ETI activate a number of overlapping downstream outputs, such as mitogen-activated protein kinases (MPKs) cascades, Ca2+ flux, hormonal signaling pathways, antimicrobial compounds, reactive oxygen and nitrogen species burst, and transcriptional reprograming (Jones and Dang 2006; Macho and Zipfel, 2014; Yuan et al., 2021).

Plant membrane pattern-recognition receptors (PRRs) recognize PAMPs to induce PTI. PRRs include receptor-like kinases (RLKs) or receptor-like proteins (RLP) with extracellular domains. More than 600 RLKs genes and 57 RLPs have been reported in *Arabidopsis* genome, and a larger number of them are involved in biotic stress response (Noman et al., 2019). After recognition, plants trigger intracellular signals such as influx of extracellular Ca2+ in the cytosol and MAPKs cascades. MAPK activation leads to phosphorylation of several transcription factors that regulates a high number of genes such as those involved in salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signaling as well as antimicrobial compound production (Nishad et al., 2020). Plant hormones regulate physiological processes during development, reproduction, and responses to biotic and abiotic factors (Taiz and Zeiger, 2014). SA, JA and ET are related to defense mechanisms against pathogens and different types of abiotic stress, while abscisic acid (ABA) is mainly related with stomatal closure (Taiz and Zeiger, 2014). The SA pathway is activated against bacterial pathogens and biotrophic fungi, while JA is generally activated against insects and necrotrophic fungi. Commonly, the SA and JA

pathways are antagonistic, while there is synergism between the JA and ET pathways (Pandey and Senthil-Kumar, 2017). ABA modulates the response of plants to pathogens by its interaction with the SA and JA / ET pathways (Pandey and Senthil-Kumar, 2017). Hormonal levels increase after pathogen colonization in plants (Studham and MacIntosh, 2012; Abdelsamad et al., 2019; Han and Kahmann, 2019), leading to the activation of genes with different roles in plant defense (Pieterse et al., 2012).

Several studies on soybean-pathogen interaction have been performed. After pathogen colonization, soybean induces the expression of genes involved in pathogen perception and signaling such as RLKs, RLPs, and MAPKs (McCabe et al., 2018; Lanubile et al., 2015), transcription factors (Bencke-Malato et al., 2014; Lanubile et al., 2015; McCabe et al., 2018 and Dong et al., 2018), PR genes (Upchurch et al., 2010), and genes related to the phenylpropanoid pathway (Zeng et al., 2017; Song et al., 2019; Ranjan et al., 2019). Furthermore, activation of cell wall reinforcement by incorporation of phenolic compounds has been observed in response to *P. pachyrhizi* (Lygin et al., 2009).

RNA-seq analysis provides a powerful tool to study differential gene expression patterns in plants in response to biotic and abiotic stresses. Transcriptome analysis has been used to identify differential expressed genes related to defense, leading to the understanding of complex gene regulatory networks in soybean plants responding to different pathogens, including bacterial leaf pustule (*Xanthomonas axonopodis* pv. *glycines*) (Kim et al., 2011; Kim et al., 2015), *Phytophthora* root and stem rot (*Phytophthora sojae*) (Lin et al., 2014), Asian soybean rust (*Phakopsora pachyrhizi*) (Tremblay et al., 2011; Benche-Malato et al., 2014), common cutworm (*Spodoptera litura*) (Wang et al., 2014), *Fusarium oxysporum* (Lanubile et al., 2015), bean pyralid larvae (*Lamprosema indicate*) (Zeng et al., 2017), soybean downy mildew (*Peronospora manshurica*) (Dong et al., 2018), *Canophora gregata* (McCabe et al., 2018), soybean cyst nematode (*Heterodera glycines*) (Song et al., 2019), soybean mosaic virus (Zhang et al 2019) and *Sclerotinia sclerotiorum* (Ranjan et al., 2019). However, transcriptomic information during *D. caulivora-G. max* interactions is not available. To get more insights into the activation of plant defenses against *D. caulivora*, we performed RNA-seq profiling of two contrasting *G. max* genotypes during *D. caulivora* infection.

Materials and methods

Plant materials and *D. caulivora* inoculation

A *D. caulivora* isolate (strain D57), collected from canker lesions of soybean in Uruguay during 2015 was used in this study (Mena et al., 2020). Two soybean genotypes were used for all plant assays: SSC-susceptible Williams PI548631 obtained from USDA ARS Soybean germplasm collection (seed source 13U-9280, order 253444, 2014); and the SSC-moderately resistance Genesis-5601 from Instituto Nacional de Investigacion Agropecuaria (INIA) breeding program. For both genotypes three soybean seeds were planted in a 10-cm-diameter pot filled with a mix of soil and vermiculite at a rate of 3:1. Soybean seedlings were grown in a growth room under a 16 h light/8 h dark lighting regime at 24°C. For all experiments, 3-week-old plants at V2 were used. *D. caulivora* D57 isolate was inoculated using the stem wounding method where an agar plug containing mycelium was applied to the wounded stem (Mena et al., 2020). As a control an agar plug without mycelium was used.

Development of SSC symptoms were compared between both soybean genotypes. Ten plants were used per treatment and the experiment was repeated three times. Lesion length (mm) and disease severity was determined at various time points [3, 5, 7, 11, and 14 days post-inoculation (dpi)]. Disease severity index, infection index and area under disease progress curve (AUDPC) was calculated according to Mena et al., 2020. Significant differences between treatments were determined by non-parametric Kruskal–Wallis and Mann– Whitney tests using SPSS Statistics v. 21.0. The significance level for data used was $p < 0.01$.

Quantitative PCR

After soybean stem inoculation with *D. caulivora*, fungal DNA was quantified at 0, 8, 24, 48, 72, and 96 hpi. Three plants per treatment were used as biological replicates and samples were frozen in liquid nitrogen. DNA extraction, quantitative PCR (qPCR), estimation of pathogen biomass and the amount of soybean DNA was performed accord to Mena et al. (2020). For qPCR, primers designed for the elongation factor gene of soybean and the b-tubulin gene of *D. caulivora* were used. Result was expressed as ng of *D. caulivora*/ng of soybean tissue. Student's t-test was applied to all qPCR data, and values of $p \leq 0.01$ were considered statistically significant.

RNA extraction, cDNA library preparation and sequencing

For transcriptomic analysis, samples of both genotypes were taken at 0 (without treatment), and at 8 and 48 hours post inoculation (hpi) with *D. caulivora* and their respective controls (plugs without mycelium). Total RNA was extracted and purified from 100 mg soybean stems, 10 mm above the inoculation point of each sample, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and Invitrogen PureLink RNA Extraction Mini kit (Invitrogen, USA), including an on-column digestion with RNase-Free DNase I, according to the manufacturer's instructions. Quality of the isolated RNA was checked by running samples on 1.2% formaldehyde agarose gel. RNA concentration was measured using a NanoDrop 2000c (Thermo Scientific, Wilmington, USA). RNA quality control, library preparation, and sequencing were performed at Macrogen Inc. (Seoul, Korea). Three biological replicated were included per treatment. Libraries for each biological replicate were prepared for paired-end sequencing by TruSeq Stranded Total RNA LT Sample Prep Kit (Plant) with 1 g input RNA, following the TruSeq Stranded Total RNA Sample Prep Guide, Part # 15,031,048 Rev. E. Sequencing was performed on Illumina platform (Illumina, CA, USA) to generate paired-end 101 bp reads, obtaining 41.6 to 65.2 M reads per sample with Q20 > 98% and Q30 > 95%.

Pre-processing of raw data, mapping of reads and annotation

All raw RNA-Seq read data are deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/) under the BioProject ID: PRJNA717275

RNA-seq processing steps were done through Galaxy platform (https://usegalaxy.org/). Raw reads quality was subjected to a quality control check using FastQC software ver. 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequences were trimmed, and the adapters removed using Trimmomatic Version 0.38.0 software (Bolger, et al. 2014). Additionally, to the default options, the following parameters were

adjusted: adapter sequence TruSeq3 (paired-ended (PE), for MiSeq and HiSeq), always keep both reads of PE, and SLIDINGWINDOW: 4:15 HEADCROP: 13 MINLEN:50. Trimmed reads were mapped to reference genome of *Glycine max* Gmax_275_Wm82.a2.v1.fa (Schmutz et al., 2010) as the reference genome file and Gmax_275_Wm82.a2.v1.gene.gff3 as a reference file for annotation gene models from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) using Hisat2 software (Kim et al., 2015). The BAM files were obtained with Samtools View software ver.1.9 and then sorted by name with Samtools Sort software ver. 2.0.3 (Li et al., 2009), for further analysis. Reads were counted using FeatureCounts software ver. 1.6.4 (Liao et al., 2013). Additionally, to default options, parameters were adjusted for: count fragments instead of reads, allow read to map to multiple features, count multi-mapping reads/fragments and use reference sequence file *Glycine max* v4.2.

Transcript expression and functional analysis

Cluster analysis of replicates from each time point and control samples were performed by Principal Component Analysis (PCA) using log2 fold changes for all datasets. Differential expression analyses were performed using EdgeR software ver. 3.24.1 (Robinson et al 2009), with p-value adjusted method of Benjamini and Hochberg adjusted threshold 0.05, (Benjamini et al 1995) and Minimum log2 Fold Change 2. Counts were normalized to counts per million (cpm) with the TMM method and low expressed genes filtered for count values ≥ 3 in all samples. In this study, a false discovery rate (FDR) ≤ 0.05 was used to determine significant differentially expressed genes (DEGs) between *D. caulivora* inoculated plants and mock; and expression values were represented by log2 ratio. Heat maps were generated using the Heatmapper server (www.heatmapper.ca/expression).

Gene ontology (GO) and functional annotations were assigned with the Blast2GO, through Omicbox software (https://www.biobam.com/omicsbox). (Conesa and Götz et al., 2008). Gene models were compared with several databases (NCBI nonredundant protein database, GO, and InterpoScan) with BlastP finding single hit at an e-value threshold of e-value ≤ 1.0E-3 using taxIds for Viridiplantae. InterproScan analysis was used to identify domains in the genome (Zdobnov and Apweiler, 2001) DEG functional enrichment analysis was performed using OmicBox software. GO terms with a FDR ≤0.05 were considered for our analysis. DEGs of each dataset were divided into upregulated and downregulated subsets. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of all DEGs were obtained through Omicbox software for *D. caulivora* inoculated vs. control samples in both soybean genotypes. All heat maps were generated using the Heatmapper server (www.heatmapper.ca/expression).

Results and discussion

Williams (susceptible) and Genesis 5601 (resistant) genotypes were inoculated with *D. caulivora* and infection progression was monitored during 14 dpi. *D. caulivora* produced symptoms of SSC disease in both soybean genotypes. Initial symptoms appeared as light brown lesion surrounding the point of inoculation in steams at 8 hpi in both genotypes. Lesion length was evaluated in soybean stems at 3, 5, 7, 11 and 14 dpi. After 3 dpi brown discoloration of the stem became darker in both genotypes. No differences in lesion length were observed at 3 and 5 dpi. Lesions expanded in both directions of the stem and showed withered leaves above de canker lesion (**Figure 1a**). Symptom development

was clearly visible at 7 dpi with a range of lesion length of 25-60 mm. At this time point, lesions in Williams were 30% larger than in Genesis 5601. At 11 dpi, Williams soybean stems were necrotic with a lesion average of 77 mm. All susceptible Williams plants were death at 14 dpi with a lesion average of 100 mm, while Genesis 5601 plants showed a range of 50-80 mm of lesions and a general healthy state (**Figure 1b**). Disease severity index and AUDPC were evaluated in soybean stems until 14 dpi and according to the disease severity scale, *D. caulivora* progress more in Williams than in Genesis 5601 during time (**Figure 1c-d**). These results indicate that lesion length, disease severity index and AUDPC increase in Williams during time, while disease progression stopped in Genesis 5601 at 11 dpi.

D. caulivora was quantified on soybean stems in both genotypes by quantitative PCR (qPCR). Fungal DNA (ng *D. caulivora*/ng soybean) was measured at 8, 24, 48, 72 and 96 hpi. As expected, pathogen biomass was higher in Williams compared to Genesis 5601 at 24 to 96 hpi. The highest quantity of the pathogen was detected at 96 hpi, with 40 ng and 10 ng of fungal tissue per 100 ng of plant tissue in Williams and Genesis 5601, respectively (**Figure 1e**). These results, together with previous results showing increased expression of defense genes during *D. caulivora* infection of Williams plants at 24-48 hpi (Mena et al., 2020), indicate that time point between 8 and 48 hpi represent early infection stages of *D. caulivora*.

Transcriptome profiling of contrasting genotypes infected with *D. caulivora*

To identify molecular players involved in early stages of soybean defense, we performed transcriptional profiling between Williams and Genesis 5601 during *D. caulivora* infection and control conditions. Soybean stem samples were taken from plants at 0 hours and from *D. caulivora*-inoculated and control plants at 8 and 48 hpi. A total of 737 million reads were generated after removing adapter sequences and low-quality reads. Reads mapped uniquely to *G. max* nuclear genome were consider for further analyses (18.2-34.5 million reads), with 82.94-97.86% of reads mapping to the reference genomes of soybean (**Table S1**). The PCA of the different treatments showed a clear separation and variability between biological replicates was very low as indicated (**Figure 2a**).

A comparative analysis of differentially expressed genes (DEGs) was performed at 0, 8 and 48 hpi in both soybean genotypes. We identified 164 DEGs at 0 hpi; 73 were upregulated and 91 were downregulated. Furthermore, a total of 2384 DEGs with different expression profiles were identified in inoculated versus control plants within genotypes. Numbers of upregulated and downregulated genes increase at 48 hpi respect to 8 hpi in both genotypes, and more upregulated genes than downregulated were observed at both time points. In total, Williams has 513 and 1342 upregulated DEGs at 8 and 48 hpi, and Genesis 5601 has 1121 and 1207 DEGs at 8 and 48 hpi, respectively (**Figure 2b, Table S2**). In total, 167 upregulated DEGs were in common in both genotypes at both time points, and some genes were uniquely expressed in one condition (**Figure 2c**). Common upregulated genes in *D. caulivora* inoculated plants encoded proteins involved in the biosynthesis of metabolites and defense proteins (26%), transcription factors (10%), receptor and proteins kinases (5%), and hormonal signaling (3%). However, 75 and 326 upregulated DEGs were only found in Williams at 8 hpi and 48 hpi, and 401 and 191 upregulated DEGs were only present in Genesis 5601 at 8 hpi and 48 hpi, respectively (**Figure 2c**). These results show a clear differential

expression profile between the resistant and susceptible soybean genotypes in response to *D. caulivora*. Only few DEGs related to photosystem and photosynthesis were downregulated upon *D. caulivora* inoculation in both genotypes, which is consistent with the fact that *D. caulivora* is a stem pathogen and stem tissues have fewer chloroplasts compared to leaves.

At 0 hpi, ten genes (14%) encoding for proteins with roles in perception and signaling were upregulated in Genesis 5601 compared to Williams, including LRR receptor-like serine/threonine-protein kinases, LRR containing proteins, receptor-like protein EIX2, and disease resistance RPP13-like protein. Moreover, 24 genes (33%) related to plant defense, hormone signaling, transcription factors and transporters were upregulated in Genesis 5601 respect to Williams. Interestingly, we identified several genes that encoding for PR-1, WRKY transcription factor ethylene-responsive transcription factor, chalcone synthase, dirigent protein, chitinase (PR-3), β-1,3 glucanase (PR-2), MYB transcription factor and TMV resistance protein, that were also induced in both genotypes at 48 hpi after *D. caulivora* inoculation. These results indicate that at a basal state, Genesis 5601 has higher gene expression levels of genes related to defense mechanisms that might play a role as a first line of resistance against *D. caulivora* infection. Furthermore, a higher number of DEGs identified in the resistant Genesis 5601 genotype compared to the susceptible Williams, which suggested that more genes are induced to resist *D. caulivora* infection in Genesis 5601, principally at 8 hpi.

Gene ontology enrichment and KEGG pathways analyses

We further focused on upregulated genes in the different comparisons by analyzing Gene Ontology (GO) term enrichment analysis and manual inspection in order to identify biological processes (BP) and molecular function (MF). Enriched upregulated BP at 0 hpi include terms related to protein phosphorylation, signal transduction, regulation of transcription, ethylene-activated signaling pathway and others biosynthetic process (**Figure 3a**). MF at 0 hpi is mostly represented by protein, ADP, ATP and DNA binding, protein kinase, oxidoreductase, acyltransferase activity and others (**Figure 3b**).

Most of the top 25 significantly enriched GO terms in inoculated vs control soybean plants were similar at 8 and 48 hpi. In BP, protein phosphorylation and regulation of transcription were most represented in both genotypes at both times. Genesis 5601 showed a greater number of genes per category respect to Williams at both times. At 8 hpi, top GO terms enrichment in BP included ethylene-activated signaling pathway, defense response, response to oxidative stress and hydrogen peroxide transmembrane transport, response to heat and salt stress, ABA activated signaling pathway, response to auxin and cell wall modification. All these GOs related to defense were also present at 48 hpi and additional upregulated defense-related GO terms included cellular oxidant detoxification, flavonoid biosynthetic process, response to biotic stimulus, bacterium, chitin and fungus (**Figure 3**). In general, biotic related process, abiotic related process, hormones and secondary metabolites represent 60% of the GO biological process (BP) terms identified in the up-regulated genes. MF terms are mostly represented by ATP binding and DNA-binding transcription factor activity in both genotypes at both times. Top MF terms included heme, protein, DNA, iron ion and sequence-specific DNA binding and protein kinase, oxidoreductase, monooxygenase, peroxidase, glycosyltransferase and protein serin threonine kinase activity. At 48 hpi, calcium, metal and ion binding, and hydrolase activity were included respect to 8 hpi (**Figure 3**).

The majority of enriched KEGG pathways were related to plant defense, such as phenylpropanoid biosynthesis, flavonoid biosynthesis, flavone and flavonol biosynthesis, isoflavonoid biosynthesis, anthocyanin biosynthesis, plant hormone signal transduction, brassinosteroid biosynthesis, ABC transporters, plant-pathogen interaction and steroid hormone biosynthesis. Both genotypes shared most of the KEGG pathways, although there were more genes and pathways unique to Genesis 5601 (resistant) than to Williams (susceptible). The most enriched KEGG pathways for upregulated DEGs in Genesis 5601 were secondary metabolites biosynthesis, plant hormone signal transduction and plant-pathogen interaction. According to the functional annotation of DEGs in GO and KEGG analysis, we analyzed in more detail those genes involving in phenylpropanoid pathway, phytohormone signaling pathways, genes encoding pathogenesis related (PR), other defense proteins and transcription factors.

Hormone signaling during soybean defense against *D. caulivora*

Plant hormones, SA, JA, ET, ABA, cytokinin (CK), gibberellin (GA), auxin, and brassinosteroid (BR), conform a complex network that regulate plant resistance against pathogens (Pandey and Senthil-Kumar, 2017). GO enrichment analysis showed that auxin, ET and ABA are probably the principal phytohormones involved in defense responses against *D. caulivora*. In total, 106 DEGs involved in phytohormone pathways and signaling were upregulated during soybean colonization. Of them, 34 (32%) were related to the biosynthesis and signaling of indole-3-acetic acid (IAA), the main auxin in plants, and include genes encoding auxin influx carrier (AUX1), auxin efflux carrier (PIN), auxin signaling F-box (AFB), auxin-binding protein (ABP), auxin responsive protein (IAA), auxins responsive GH3 gene family (GH3), small auxin up RNA SAUR family (SAUR) and IAA induced protein ARG2 (ARG2). Influx and efflux carrier genes were expressed in Genesis 5601, but were downregulated in Williams at 48 hpi. While two *iaa* and three *gh3* genes were upregulated in Genesis 5601 at 48 hpi, only one *iaa* and *gh3* were upregulated in Williams. A higher number of *saur* genes were activated in Genesis 5601 at 8 hpi and in Williams at 48 hpi (**Figure 4, Table S3**). GH3 gene family can promote amino acids to be combined with IAA, JA and SA, leading to changes in the concentration of biologically active forms within the cells.

We also found 35 DEGs (33%) related to ET synthesis and signaling. ACS (1-aminocyclepropane-1-carboxylate synthase) encoding genes, which catalyzes the first step of ET biosynthesis, were upregulated only in Genesis 5601 at 8 hpi and in both soybean genotypes at 48 hpi. Eight additional DEGs coding for 1-aminocyclepropane-1-carboxylate oxidase (ACO), the second step of the ET biosynthesis, were upregulated in both soybean genotypes and mostly at 48 hpi. Interestingly, induction of ACS and ACO genes has been associated with resistance against vascular diseases in soybean resistant genotype against *Fusarium virguliforme* (Abdelsamad et al., 2019). ET receptor (ETR) was upregulated in both genotypes at 48 hpi, and Genesis 5601 exhibited higher expression levels. In addition, the number of upregulated genes encoding ET responsive transcription factor (ERF) were considerably higher in Genesis 5601 compared to Williams at 8 hpi. Furthermore, seven DEGs were related to ABA signaling. Four of them encode for receptor PYL, and three of them were upregulated at 48 hpi in Genesis 5601 and only one in Williams. One 2C protein phosphatase (PP2C) gene was upregulated in Genesis 5601 at 8 hpi, which is involved in the inhibition of the ABA signaling

transduction, suggesting suppression of ABA signaling. However, another PP2C gene was downregulated in Williams at 48 hpi, indicated activation of ABA signaling (**Figure 4, Table S3**).

We also identified ten upregulated DEGs related to CKs, including cytokinin dehydrogenases (CKX) and cytokinin hydroxylases (CYP735), being the latter only upregulated in Genesis 5601. In total, ten upregulated DEGs were involved in GA biosynthesis; a gibberellin 20 oxidase (GAox) gene, which is required for the release of bioactive forms of gibberellins, was upregulated only in Genesis 5601 at 8 hpi. Additionally, gibberellin 2-beta-dioxygenase was upregulated in Genesis 5601 at 8 hpi and in both genotypes at 48 hpi. Five DEGs were identified as gibberellin-regulated protein (GRP) and showed differential expression profile. Transcription factors related to GA, phytochrome-interactin factor (PIF) were upregulated in Genesis 5601 and downregulated in Williams at 8 hpi. Genes encoding a BR insensitive 1-associated receptor kinase and a transcription factor MYC2 involved in jasmonate signaling, were only upregulated in Williams at 48 hpi. Several genes, which could be involved in SA synthesis, were also upregulated after *D. caulivora* inoculation, including three genes encoding phenylalanine ammonia-lyase (PAL), which were upregulated in both soybean genotypes at both times, and the SA marker genes PR-1, which were upregulated at 48 hpi in both genotypes (**Figure 4, Table S3**). Taken together, these results suggest that several hormones, especially IAA, ET, SA and ABA, are involved in resistance against *D. caulivora*. Further studies are needed to understand the precise role of these hormones in soybean defense responses against this fungal pathogen.

Activation of phenylpropanoid pathways during soybean defense against *D. caulivora*

Genes related to secondary metabolites biosynthesis are important in defense, since they are involved in reinforcement of the cell wall through lignin and phenolic compounds, synthesis of SA and production of antimicrobial compounds such as flavonoids, terpenoids (Vogt, 2010; Piasecka et al., 2015). The majority of enriched KEGG pathways of the upregulated DEGs were related to phenylpropanoid biosynthesis, flavonoid biosynthesis, flavone and flavonol biosynthesis, isoflavonoid biosynthesis and anthocyanin biosynthesis. When looked into more detail to the phenylpropanoid pathway after *D. caulivora* inoculation (**Figure 5, Table S4**). In total, 196 DEGs of the phenylpropanoid pathway were identified during *D. caulivora* infection with different expression profile. DEGs were mainly induced at 8 hpi in Genesis 5601 and at 48 hpi in both genotypes. Genes encoding PAL, cinnamyl alcohol dehydrogenase (CAD), caffeic acid 3-O-methyltransferase (COMT), chalcone isomerase (CHI), Quercetin 3-O-methyltransferase (QOMT), which are responsible for the production of flavonols, were upregulated at 48 hpi in both genotypes. Several peroxidases (POD), which have a role in lignin synthesis and other phenolic compounds (Wang et al. 2013), were induced after *D. caulivora* inoculation. Only seven *pods* were upregulated in Williams at 8 hpi, while 27 were induced in Genesis 5601 at this time. Moreover, 24 and 31 *pods* were upregulated at 48 hpi in Williams and Genesis 5601, respectively. In addition, isoflavone 7-O-methyltransferase (FOMT) and flavone synthase II (FNS II), involved in flavone and isoflavone biosynthesis, were upregulated only in Genesis 5601 at 8 hpi. Furthermore, 2-oxoglutarate-dependent dioxygenase (ANS) and UDP-glycosyltransferase (IF7GT) were upregulated only at 48 hpi in both genotypes. Several isoflavone reductases (IFRs)

and vestitone reductase (VR) related to isoflavonoids biosynthesis were upregulated in both genotypes at 48 hpi and only in Genesis 5601 at 8 hpi (**Figure 5, Table S4**). These results indicate that lignification, cell wall strengthening, flavonoid and phytoalexin production are defense mechanisms activated in soybean plants during *D. caulivora* infection. The fact that a high proportion of genes of this pathway are induced at an earlier time point (8 hpi), suggest that the metabolites produced could be involved in resistant mechanisms in Genesis 5601. Consistently, upregulation of genes encoding PAL and the activity of this enzyme increased in resistant, but not in susceptible soybean cultivars after nematode infection (Edens et al 1995). The phenylpropanoid pathway leading to the synthesis of lignin and flavonoids is also induced in soybean plants inoculated with *Pseudomonas syringae, Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Zabala et al., 2006; Chen et al., 2010; Ranjan et al., 2019).

Expression of genes involved in pathogen perception, transcription and defense mechanisms during soybean infection by *D. caulivora*

Pathogen recognition and signaling are important steps in the activation of plant defense mechanism against pathogens. We identified 219 DEGs encoding PRRs and protein kinases related to signaling. PRRs included different classes of receptors such as ten leucine-rich repeat receptor-like protein kinase (LRR- RLKs), 28 receptors like protein kinases (RLKs), 19 receptors like protein (RLP), 22 LRR receptor-like serine/threonine-protein kinase (LRR-STKs), six L-type lectin-domain containing receptor kinase, six wall-associated receptor kinase-like (WAKL), 21 Cysteine-rich receptor-like protein kinase (CRKs), 18 receptor-like serine/threonine-protein kinase with lectin domain (Lec-RL-ST), two receptor-like serine/threonine-protein kinase with leucine-rich repeat domain (LRR-RL-ST), a receptor-like tyrosine -protein kinase with leucine-rich repeat domain (LRR-RL-T), four Lectin domain containing receptor kinase (LecRLKs), two lysM domain receptor-like kinase (Lys-RLKs), ten MDIS1-interacting receptor like kinase, three calmodulin-binding receptor kinase (CaMRLK), and other receptors (**Figure 6, Table S5**). In Genesis 5601, 79 and 101 receptor genes were upregulated at 8 hpi and 48 hpi respectively, while 20 genes were upregulated in Williams at 8 hpi and 199 genes were upregulated at 48 hpi. LRR-RLKs, CRKs, Lec-RL-ST, Lec-RLKs and Lys-RLKs were mostly upregulated at 8 hpi in Genesis 5601 and not in Williams, while at 48 hpi a high number of these and other PRRs were upregulated in both cultivars (**Figure 6, Table S**5). In accordance with these results, several examples show that a delay in the plant defense response led to a susceptible interaction. Early activation of plant defenses in resistant genotypes depends on a rapid recognition of pathogen elicitors. RLKs, RLPs and PRR with LRR, LysM and Lec domains are reported to play important roles in pathogen perception (Goff and Ramonell, 2007; Tang et al., 2017). RLP, RLK, NBS-LRR are associated with *Rbs* resistance loci on chromosome 16 of soybean against *Cadophora gregata* (McCabe et al., 2018). LRR-STKs were induced in resistant and susceptible soybean plants against bean pyralid (Zeng et al., 2017). Moreover, several soybean CRKs are induced in response to PAMPs such as flagelin and chitin, indicating their involvement in pathogen perception (Delgado-Cerrone et al., 2018).

DEGs encoding protein kinases included 25 Serine/threonine-protein kinase (STKs), two Serine/threonine- protein phosphatase (STPs), two CBL-interacting protein kinase (CIPKs), three MATE efflux family protein and ten mitogen-activated protein kinases (MAPKs). Several MAPKs were differentially expressed. Two of them were MAPK, three

MAPKK and five MAPKKK. Two MAPK and one MAPKK genes were upregulated in Genesis 5601 and in Williams at 48 hpi. Four MAPKKKs genes were upregulated in Genesis 5601 at 8 hpi, and only one of them was induced in Williams at 48 hpi (**Figure 6, Table S5**). MAPKs, MAPKKs and MAPKKKs are protein kinases related to pathogen recognition that trigger and activate downstream signaling (Pedley and Martin, 2005; Rodriguez et al., 2010; Zamora-Ballesteros et al.,2021). Lanubile et al. 2015 identified 3 MAPKKs induced in soybean roots in response to *Fusarium oxysporum*. Additional transcriptional analysis in soybean identified activation of MAPK gene expression and defense responses to infection with *Heterodera glycines* (McNeece et al., 2019).

Genes related to calcium signaling (26) were up and downregulated in both genotypes at both times (**Figure 7a, Table S6**). DEGs encoding transporters (38) were upregulated in both soybean genotypes at 48 hpi. ABC transporters represented the category with the highest number of genes (47%) and they were upregulated in Genesis 5601 at both times and in Williams mostly at 48 hpi (**Figure 7b, Table S6**). Furthermore, DEGs encoding defense proteins (83) were mainly upregulated at 48 hpi. 21 of them (25%) were disease resistance protein and 17 (20.5%) were PR protein, including PR-1, PR-4 and PR-10. Disease resistance protein were upregulated at 8 hpi in Genesis 5601 and in both genotypes at 48 hpi and PR were upregulated at 48 hpi in both genotypes (**Figure 8, Table S7).**

Disease resistance genes (R-genes) encode proteins involved in detecting pathogen attack and activating downstream defense molecules. Most of them contain NBS-LRR domains (Nepal and Benson, 2015). A total of 319 genes were assigned as putative NBS-LRR genes in the soybean genome (Kang et al., 2012). From them, nine genes were highly expressed at basal levels in Genesis 5601, including disease resistance RPP13, disease resistance protein RGA1, disease resistance protein RPM1, among others. En Genesis 5601 at 8 hpi, Genesis 5601 at 48hpi and Williams at 48 hpi were identified 13, 9, 13 R-genes respectively; including disease resistance RPP13, disease resistance protein RGA1, disease resistance protein RPM1 and, leaf rust 10 disease-resistance; however only two R-genes were identified in Williams at 8 hpi. Using genome-wide association studies (GWAS) some SNPs has been identified associated with bacterial, fungal, nematodes and viral diseases (Chang et al., 2016). In *Arabidopsis* plants *Rpm* confers resistance to *Pseudomonas syringae* (Boyes et al., 1998) and *Rml* to *Leptosphaeria maculans* (Staal et al., 2006). In wheat leaf rust resistance gene confers resistance to *Puccinia triticina* (Kolodziej et al., 2021). In soybean plants some R-genes has identified, like *Rpg* that confers resistance to *Pseudomonas glycinae* (Ashfield et al., 2012), *Rbs* resistance against *C. gregata* (McCabe et al., 2018), five *Rdm* against *D. aspalathi* (Chiesa et al., 2009; Pioli et al., 2003), more than 20 *Rps* genes against *P. sojae* (Zhong et al., 2018), *Rpp* genes conferring resistance against *P. pachyrhizi* (Kashiwa et al., 2020) and *Rhg* against *H. glycines* (Jiao et al., 2015).

PRs are small proteins with antimicrobial activities. PR proteins are generated and accumulated after pathogen invasions or abiotic stress, and they have a role in defense response activation (van Loon et al. 2006; Jain and Khurana, 2018). PR-1, PR-2, PR-4, PR-10 were also induced in soybean tissues during the response to *Fusarium oxysporum* and *D. caulivora* (Lanubile et al. 2015; Mena et al., 2020), after bean pyralid feeding most of the upregulated DEGs were PR (Zeng et al., 2017). Our results suggested that PR proteins are involved in the defense responses of soybean to *D. caulivora*

In addition, others genes involved in defense response were induced in both genotypes such as alpha-dioxygenase (DOX1), pathogen-associated molecular patterns-induced secreted protein or peptide (PIP). Similar as previous results, a higher number of genes encoding defense proteins were observed in Genesis 5601 compared to Williams at 8 hpi, while at 48 hpi the number of upregulated genes were similar. These include several dirigent proteins, involved in the synthesis of lignin-like compounds, and cell wall modifying enzymes such as pectinesterase/pectinesterase inhibitor (PEI), xyloglucan endotransglucosylase/hydrolase protein (XTH), cellulose synthase-like protein E1 (CSLE1) and expansins (EXPs) (**Figure 8, Table S7**).

In total 170 transcription factors DEGs were identified, most of which were upregulated. In total, 35 WRKY (20%) were upregulated and at 48 hpi DEGs increased significantly respect to 8 hpi in both genotypes. WRKY55 and WRKY61 were induced only in Genesis 5601 at 8 hpi, while WRKY24, WRKY51 and WRKY75 were upregulated in all treatments **(Figure 9, Table S8**). WRKY proteins are a large family of transcriptional regulators, activators or repressors, involved in plant responses to biotic and abiotic stresses (Jiang et al, 2017; Dong et al., 2018). In soybean, some WRKYs have been identified in resistance responses to pathogens such as *Phakopsora pachyrhizi* (Bencke-Malato et al., 2014), *Fusarium oxysporum* (Lanubile et al., 2015), bean pyralid larvae (Zeng at al., 2017), *C. gregata* (McCabe et al., 2018), *Peronospora manshurica* (Dong et al., 2018) and *Heterodera glycines* (Jiang et al., 2020). In addition, 59 DEGs (35%) encoding for ERFs were identified. A high number of them are upregulated at 8 hpi, 48 hpi or both time points. ERFs are known to be involved in ethylene signaling and their overexpression in transgenic plants can confer enhanced disease resistance against different types of pathogens (Huang et al., 2004).

On the other hand, 28 (16.5%) MYB transcription factor, three basic leucine zipper (bZIP) transcription factor, 17 bHLH transcription factor and others ten transcription factors DEGs were identified (CPC, KAN, MYC, ORG, PIF, RAX and TRY) with different expression profile. bZIP transcription factor were induced in both genotypes, only at 48 hpi. bZIPs belong to a plant-specific transcription factor family that plays crucial roles in response to biotic and abiotic stresses (He et al., 2020). Overexpression of a bZIP from soybean in *Arabidopsis* showed increased resistance to *S. sclerotiorum* and *Pseudomonas syringae* associated with upregulated ABA-, JA-, ETH- (ethephon-)and SA-induced marker genes expression (He et al., 2020). In addition, five MYB and bHLH transcription factor genes were induced in all treatments, most of them were induced at 8 hpi in Genesis 5601 and at 48 hpi in both genotypes (**Figure 9, Table S8**). In soybean plants inoculated with *P. pachyrhizi*, a high proportion of DEGs contained MYBs associated with the stress response (Tremblay et al., 2011). MYB, AP2/ERF and ERF are associated with biotic and abiotic stresses. Glyma.10G036700, Glyma.10G186800 and Glyma.20G203700 (ERFs) were induced in stems of resistant and susceptible soybean genotypes against *C. gregata* (McCabe et al., 2018), and the genes were upregulated in all treatments, except Glyma.10G036700 that was not upregulated in Williams at 8 hpi. bHLH are involved in essential physiological and developmental processes by binding to E- and G-box sequences in the promoters of stress-response genes (Cheng et al., 2018). bHLH are also induced in soybean isogenic lines and Williams during *P. sojae* inoculation (Lin et al., 2014), and expression levels of bHLH is higher in resistant than in susceptible soybean cultivar (Cheng et al., 2018).

Cluster of gene expression analysis in soybean plants during *D. caulivora* infection

As mentioned previously, Genesis 5601 exhibited a significantly higher number of upregulated DEGs compared to Williams at 8 hpi, 1121 versus 513, suggesting a faster response. Since timing in the activation of defense reactions can determine if a genotype is resistant or susceptibility, we performed a more detailed analysis.

Hierarchical clustering grouped genotypes per time of evaluation. Genesis 5601 8 hpi was more related to 48 hpi of both genotypes and separated to Williams 8 hpi (**Figure 10a, Table S9**), which is consistent with the higher number of DEGs. Of the total 2384 DEGs, differentially expression patterns separated samples into six cluster: Cluster 1 and cluster 2 included DEGs only upregulated at 8 hpi and 48 hpi respectively in the William genotype, cluster 3 grouped genes downregulated in Genesis 5601 at 48 hpi, cluster 4 and 5 included DEGs only upregulated in Genesis 5601 at 8 and 48 hpi respectively, and finally cluster 6 grouped genes upregulated in both genotypes at 48 hpi, and in conjunct with Genesis 5601 at 8 hpi and downregulated genes only expressed in Williams at 8hpi.

We focused on the 400 genes only upregulated at 8 hpi in Genesis 5601 during the early response of *D. caulivora* infection. BP enrichment analysis of these genes were related to response to regulation of transcription, protein phosphorylation, response to heat, protein folding and complex oligomerization, response to salt stress, response to hydrogen peroxide and to oxidative stress, ethylene-activated signaling pathway, transmembrane transport, cellular oxidant detoxification, protein ubiquitination, defense response and auxin-activated signaling pathway. In addition, MF enrichment genes of these genes were related to ATP binding, DNA-binding transcription factor activity, metal ion binding, heme binding, protein serine/threonine kinase activity, DNA binding, unfolded protein binding, protein self-association, sequence-specific DNA binding, protein serine kinase activity, hydrolase activity, oxidoreductase activity, peroxidase activity, monooxygenase activity, protein dimerization activity, glycosyltransferase activity and glutathione transferase activity (**Figure 10b**). In general, the presence of categories such as response to ROS, defense responses and phytohormone signaling pathway was not surprising. Most of these categories are related to different mechanisms involved in gene regulation associated with defense responses to *D. caulivora*.

When we looked into more detail to the genes within these categories, we identified in Genesis 5601 a high percent of unique genes represented by hypothetical and uncharacterized protein (10.5%), receptor and protein kinases (8%), transcription factors (7.5%), genes of the phenylpropanoids pathway (7%), genes involved in response to hydrogen peroxide and oxidative stress (5%), and genes involved in hormone signaling (4%). The exclusively upregulation of several genes involved in auxin, ET and ABA signaling support the role of these hormones in resistance against *D. caulivora*. Taken together, these results suggest that the fast induction of defense related genes in Genesis 5601, most of which were also induced at 48 hpi in both genotypes, is responsible for the resistant phenotype. These findings present useful knowledge for the development of breeding programs to manage SSC.

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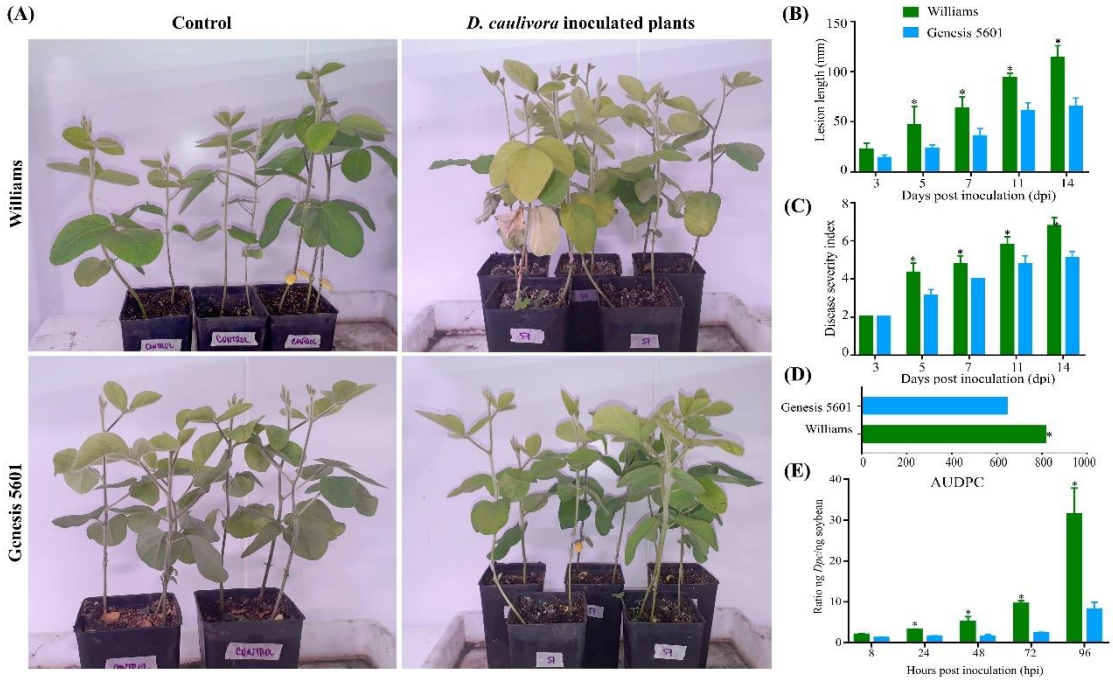
Author Contributions

EM performed all the experiments, interpreted the data, contributed to discussions, and helped to write the manuscript. SS contributed to discussions, and helped to write the manuscript. MM helped to design and to supervise the study and helped to write the manuscript. IP designed and supervised the study, interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript.

Figures



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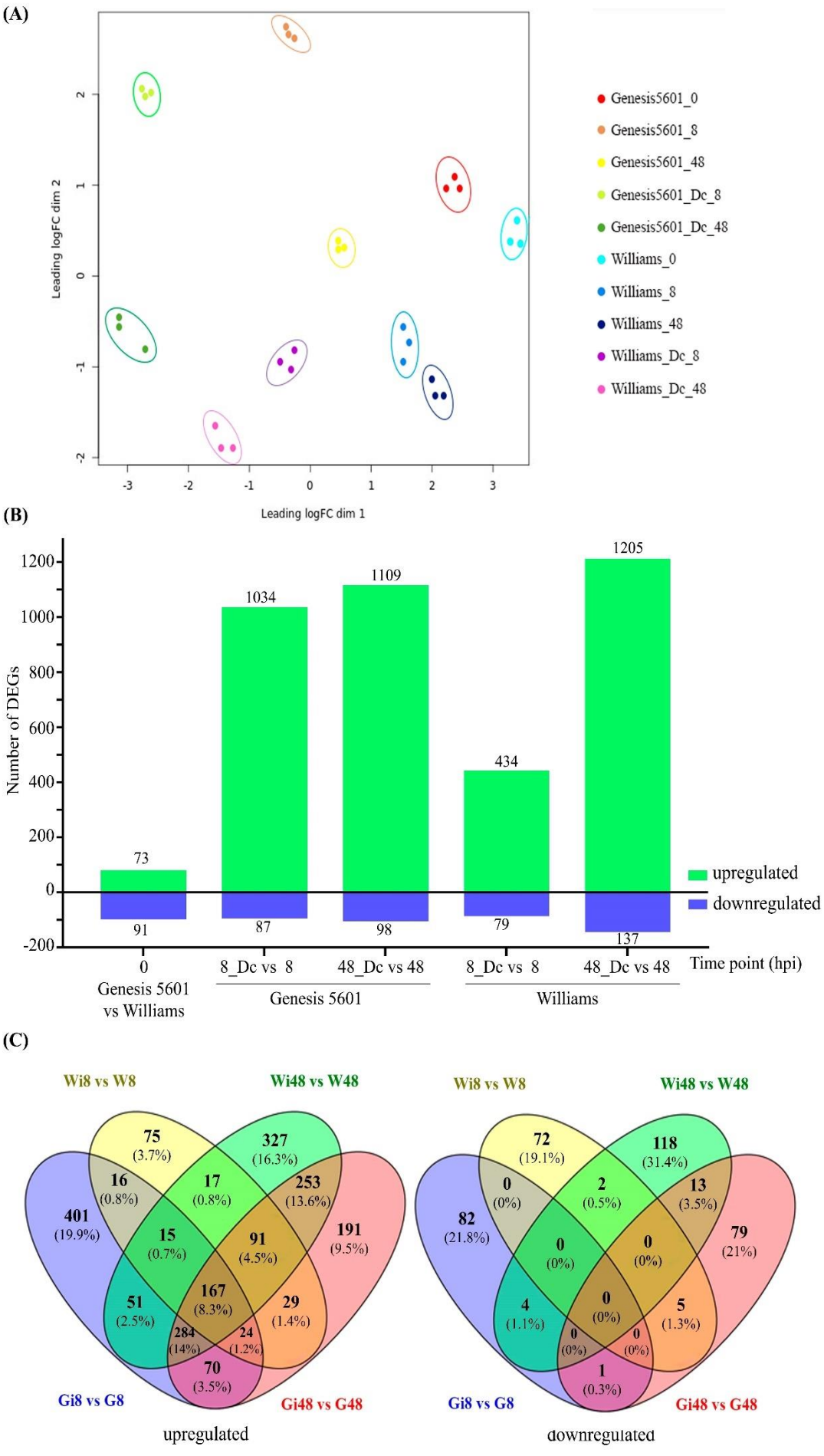
819 **Figure 1 SSC disease progress after *D. caulivora* inoculation.** A) Symptoms in susceptible
820 and resistant soybeans observed following *D. caulivora* inoculation at 7 days post-
821 inoculation (dpi). B) Lesion length in susceptible and resistant soybeans at 3, 5, 7, 11 and
822 14 dpi. C) Disease severity index in susceptible and resistant soybeans at 3, 5, 7, 11 and
823 14 dpi. D) AUDPC in susceptible and resistant soybeans at 3, 5, 7, 11 and 14 dpi. *D.*
824 *caulivora* biomass in susceptible and resistant soybeans at 8, 24, 48, 72 and 96 hpi. *
825 Indicates a significant difference between the soybean genotypes at p-value < 0.05
826 (One-way ANOVA).


(A)
Leading logFC dim 2
Leading logFC dim 1
Genesis5601_0
Genesis5601_8
Genesis5601_48
Genesis5601_Dc_8
Genesis5601_Dc_48
Williams_0
Williams_8
Williams_48
Williams_Dc_8
Williams_Dc_48
(B)
Number of DEGs
upregulated
downregulated
73
91
1034
87
1109
98
434
79
1205
137
0
Genesis 5601
vs Williams
8_Dc vs 8
48_Dc vs 48
Genesis 5601
8_Dc vs 8
48_Dc vs 48
Williams
Time point (hpi)
(C)
Wi8 vs W8
Wi48 vs W48
Gi8 vs G8
Gi48 vs G48
upregulated
75 (3.7%)
327 (16.3%)
16 (0.8%)
17 (0.8%)
253 (13.6%)
401 (19.9%)
15 (0.7%)
91 (4.5%)
191 (9.5%)
167 (8.3%)
51 (2.5%)
29 (1.4%)
284 (14%)
24 (1.2%)
70 (3.5%)
Wi8 vs W8
Wi48 vs W48
Gi8 vs G8
Gi48 vs G48
downregulated
72 (19.1%)
118 (31.4%)
0 (0%)
2 (0.5%)
13 (3.5%)
82 (21.8%)
0 (0%)
0 (0%)
79 (21%)
0 (0%)
4 (1.1%)
5 (1.3%)
0 (0%)
0 (0%)
0 (0%)
1 (0.3%)

827

Figure 2 Differentially expressed genes (DEGs) identification in susceptible and resistant soybeans at 0 hpi and after *D. caulivora* infection. A) Two-dimensional scatterplot of the principal component analyses (PCA) for soybeans where distances approximate the typical log2 fold changes between the samples. Colored dots denote each biological replicate. B) Number of DEGs for each treatment in both genotypes. Log2 FC ≥ 2.0 or ≤ 2.0 and false discovery rate (FDR) ≤ 0.05 were considered for DEGs identification. C) Venn diagram showing the number of soybean genes upregulated and downregulated at 8 and 48 hpi in susceptible and resistant soybeans. Overlap of expressed fungal genes can be observed.

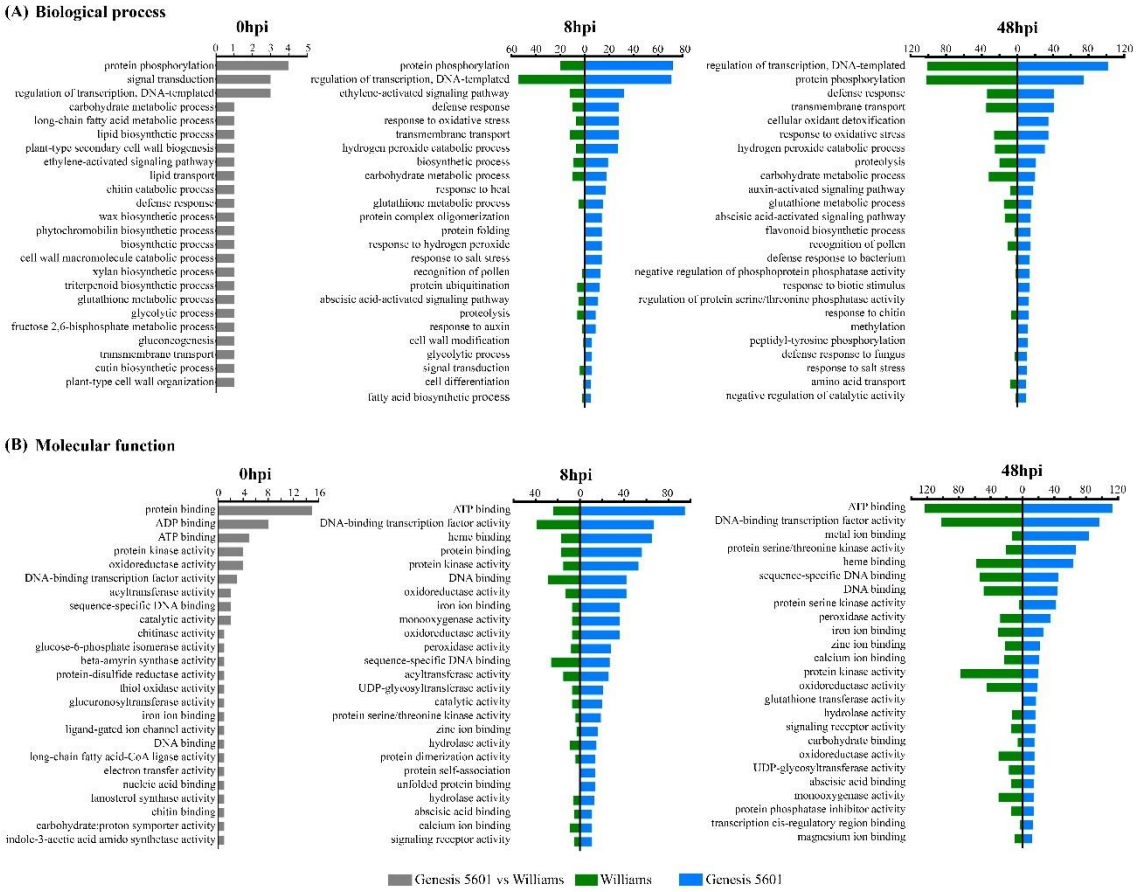


Figure 3 Enriched Gene Ontology terms. Top 25 of most significantly ($p < 0.05$) upregulated genes of Williams (green bars) and Genesis 5601 (blue bars) at 0 hours without treatments, 8 and 48 hours post inoculated with *D. caulivora*. A) Biological processes and B) Molecular function

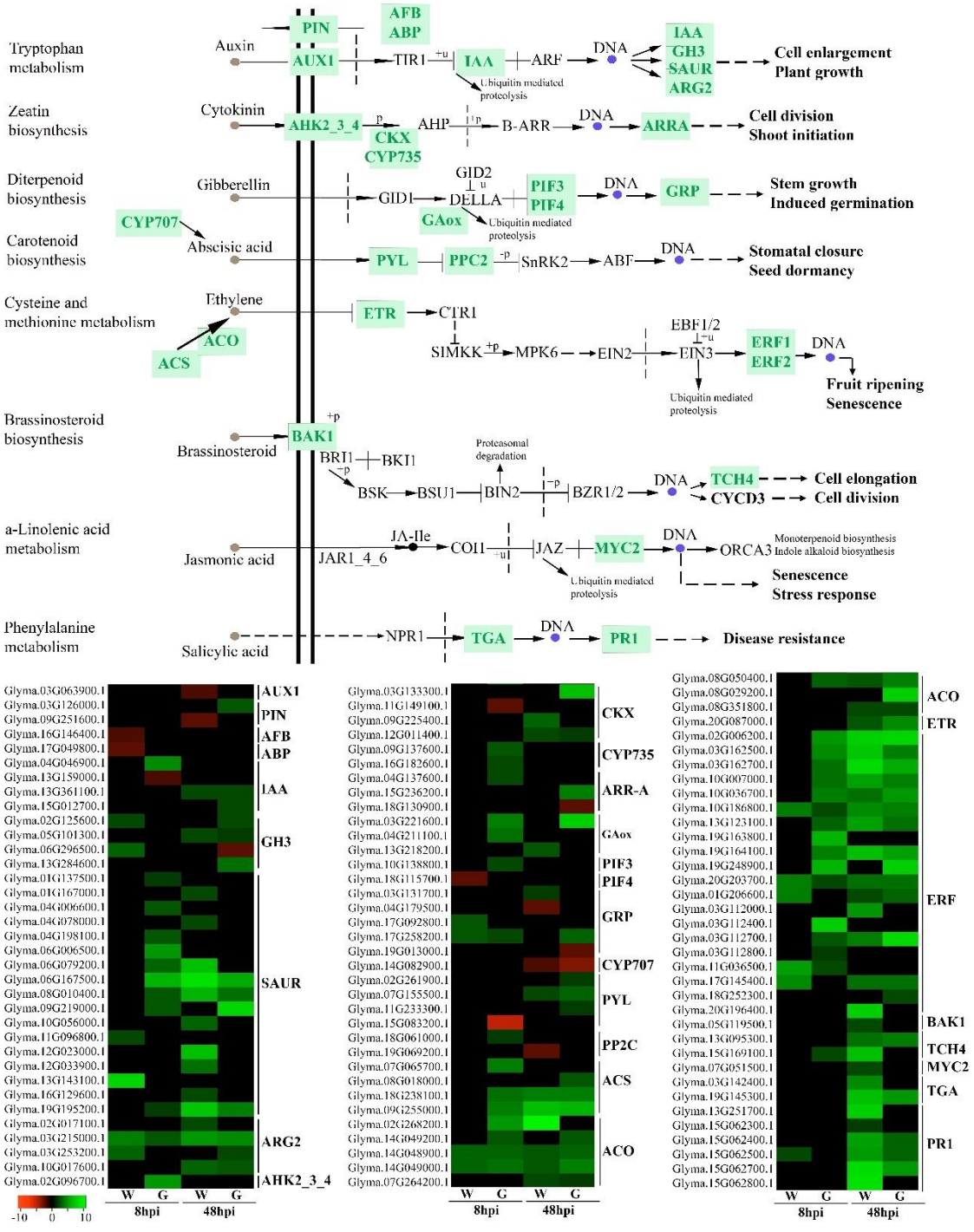


Figure 4 A) Simplified scheme of phytohormones signaling. Upregulated (green) genes are represented. B) Heatmap of differentially expressed genes (DEGs) encoding enzymes of defense hormone signaling. Individual genes are listed and colors represented the log2 fold change value based on the comparison of the transcript levels between *D. caulivora* inoculated and control. Green (upregulated), Red (downregulated), abbreviated as follows: auxin influx carrier (AUX1), auxin efflux carrier (PIN), auxin signaling F-box (AFB), auxin-binding protein (ABP), auxin responsive protein (IAA), auxin responsive GH3 gene family (GH3), SAUR family protein (SAUR), Indole-3-acetic acid-induced protein ARG2 (ARG2), Arabidopsis histidine kinase 2/3/4, a Cytokinin receptor (AHK2_3_4), cytokinin dehydrogenase (CKX), cytokinin hydroxylase (CYP735), Two-component response regulator ARR-A family (ARR-A), gibberellin oxidase (GAox),

Phytochrome-interactin factor 3 (PIF3), phytochrome-interactin factor 4 (PIF4), gibberellin-regulated protein (GRP), abscisic acid 8'-hydroxylase (CYP707), abscisic acid receptor PYR/PYL family (PYL), Protein phosphatase 2C (PP2C), 1-aminocyclopropane-1-carboxylate synthase (ACS), 1-aminocyclopropane-1-carboxylate oxidase (ACO), Ethylene receptor (ETR), Ethylene-responsive transcription factor (ERF1), Ethylene-responsive transcription factor 1 (ERF1), Ethylene-responsive transcription factor 2 (ERF2), Brassinosteroid Insensitive 1-associated receptor 1 (BAK1), Xyloglucan:xyloglucosyl transferase (TCH4), Transcription factor MYC2 (MYC2), Transcription factor TGA (TGA), Pathogenesis-related protein 1 (PR-1).

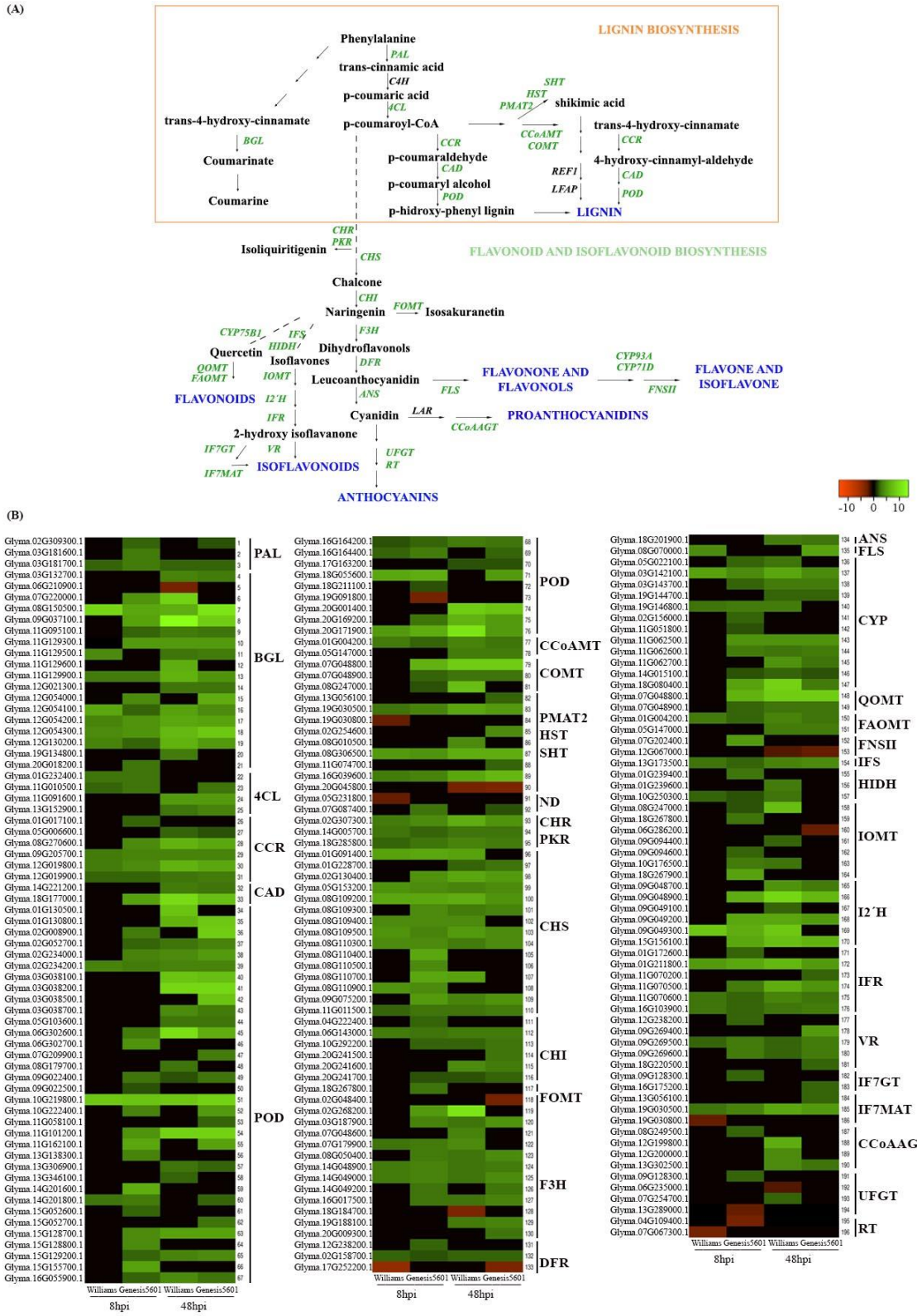
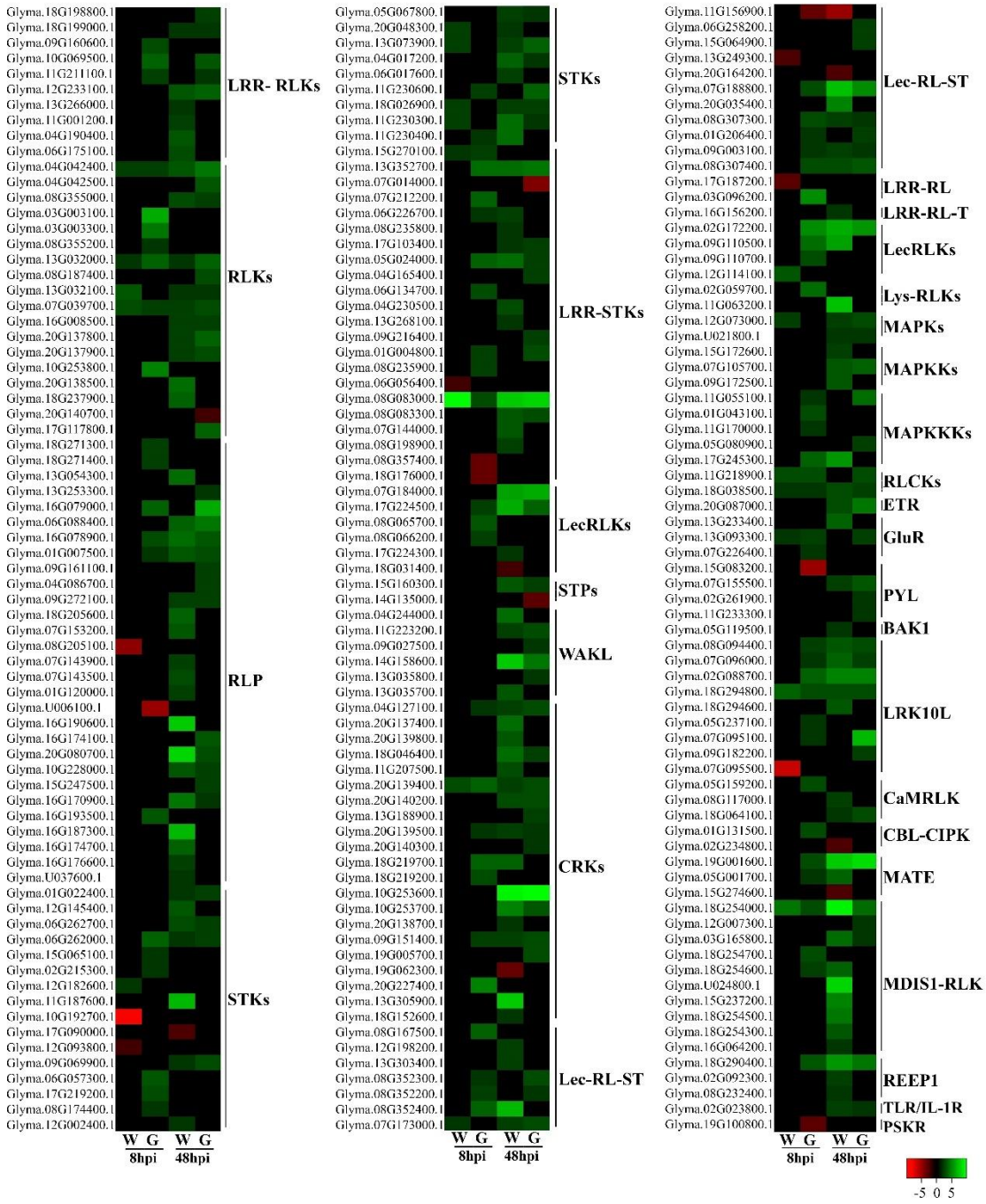


Figure 5 A) Simplified scheme of phenylpropanoid biosynthetic pathway. Some critical upregulated (green) enzymes are represented. B) Heatmap of differentially expressed genes (DEGs) encoding genes of phenylpropanoid biosynthetic pathway. Individual genes are listed and colors represented the log2 fold change value based on the comparison of the transcript levels between *D. caulivora* inoculated and control. Green (upregulated), Red (downregulated), abbreviated as follows: phenylalanine ammonia-lyase (PAL), beta-glucosidase (BGL), 4-coumarate--CoA ligase (4CL), cinnamoyl-CoA

reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), peroxidase (POD), Caffeoyl-CoA O-methyltransferase (CCoAMT), caffeic acid 3-O-methyltransferase (COMT), phenolic glucoside malonyltransferase 1-like (PMAT2), spermidine hydroxycinnamoyl transferase (HST) Shikimate O-hydroxycinnamoyl transferase (SHT), chalcone reductase (PKR), chalcone synthase (CHS), chalcone isomerase (CHI), isoflavone 7-O-methyltransferase (FOMT), flavanone 3-hydroxylase (F3H), dihydroflavonol-4-reductase (DFR), 2-oxoglutarate-dependent dioxygenase (ANS), DMR6-LIKE OXYGENASE 2 (FLS), Cytochrome P450 (CYP), Quercetin 3-O-methyltransferase (QOMT), flavonoid 3',5'-methyltransferase-like (FAOMT), flavone synthase II (FNSII), isoflavone synthase 2 (2-IFS), 2-hydroxyisoflavanone dehydratase (HIDH), isoflavone 7-O-methyltransferase (IOMT), Isoflavone 2'-hydroxylase (I2´H), isoflavone reductase (IFR), vestitone reductase (VR), UDP-glycosyltransferase (IF7GT), phenolic glucoside malonyltransferase (IF7MAT), coumaroyl-CoA:anthocyanidin 3-O-glucoside-6''-O-coumaroyltransferase (CCoAAGT), UDP-glycosyltransferase (31-UFGT), putative UDP-rhamnose:rhamnosyltransferase (RT), no data (ND)



887

888 **Figure 6 Heatmap of differentially expressed genes (DEGs) encoding for proteins with**
889 **role in perception and signaling.** Individual genes are listed and colors represented the
890 log2 fold change value based on the comparison of the transcript levels *between D.*
891 *caulivora* inoculated and control. Green (upregulated), Red (downregulated),
892 abbreviated as follows: leucine-rich repeat receptor-like protein kinase (LRR- RLKs),
893 receptor like protein kinases (RLKs), receptor like protein (RLP), Serine/threonine-
894 protein kinase (STKs), LRR receptor-like serine/threonine-protein kinase (LRR-STKs), L-
895 type lectin-domain containing receptor kinase, Serine/threonine-protein phosphatase
896 (STPs), wall-associated receptor kinase-like (WAKL), Cysteine-rich receptor-like protein
897 kinase (CRKs), receptor-like serine/threonine-protein kinase with lectin domain (Lec-RL-
898 ST), receptor-like serine/threonine-protein kinase with leucine-rich repeat domain (LRR-
899 RL-ST), receptor-like tyrosine-protein kinase with leucine-rich repeat domain (LRR-RL-T),

Lectin domain containing receptor kinase (LecRLKs), lysM domain receptor-like kinase (Lys-RLKs), mitogen-activated protein kinase (MAPKs), mitogen-activated protein kinase kinase (MAPKKs), mitogen-activated protein kinase kinase kinase (MAPKKKs), Receptor-like cytoplasmic kinase (RLCKs), ethylene receptor (ETR), glutamate receptor (GluR), abscisic acid receptor (PYL), brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1), leaf rust 10 disease-resistance locus receptor-like protein kinase (LRK10L) calmodulin-binding receptor kinase (CaMRLK), CBL-interacting protein kinase (CBL-CIPK), MATE efflux family protein (MATE), MDIS1-interacting receptor like kinase (MDIS1-RLK), receptor expression-enhancing protein (REEP1), a toll/interleukin-1 receptor-like protein TLR/IL-1R, phytosulfokine receptor (PSKR).

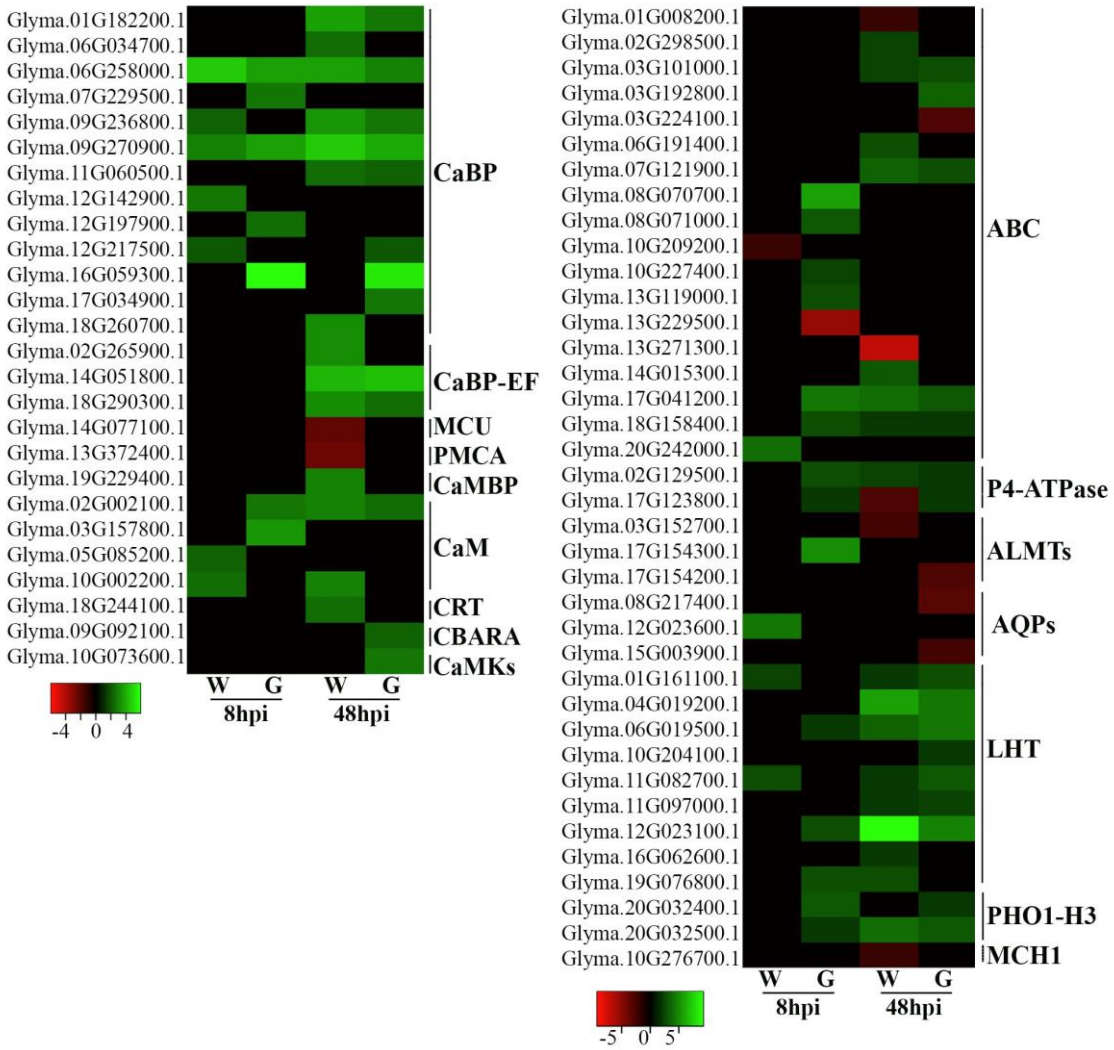


Figure 7 Heatmap of differentially expressed genes (DEGs) related to Calcium signal and transporters. Individual genes are listed and colors represented the log2 fold change value based on the comparison of the transcript levels between *D. caulivora* inoculated and control. Green (upregulated), Red (downregulated), abbreviated as follows: calcium-binding protein (CaBP), calcium-binding EF-hand protein (CaBP-EF), calcium uniporter protein 4 (MCU), calcium-transporting ATPase 9 (PMCA), calmodulin-binding protein 60 D isoform X1 (CaMBP), calmodulin-like protein 1 (CaM) and Calreticulin-3 isoform B (CRT), Calcium binding atopy-related autoantigen (CBARA), calcium/calmodulin-dependent protein kinase (CaMKs), ABC transporter (ABC), phospholipid-transporting ATPase (P4-ATPase), Aluminum-activated malate transporter

(ALMTs), aquaporin (AQPs), lysine histidine (LHT), phosphate transporter PHO1 homolog 3 (PHO1-H3), transporter MCH1 (MCH1).

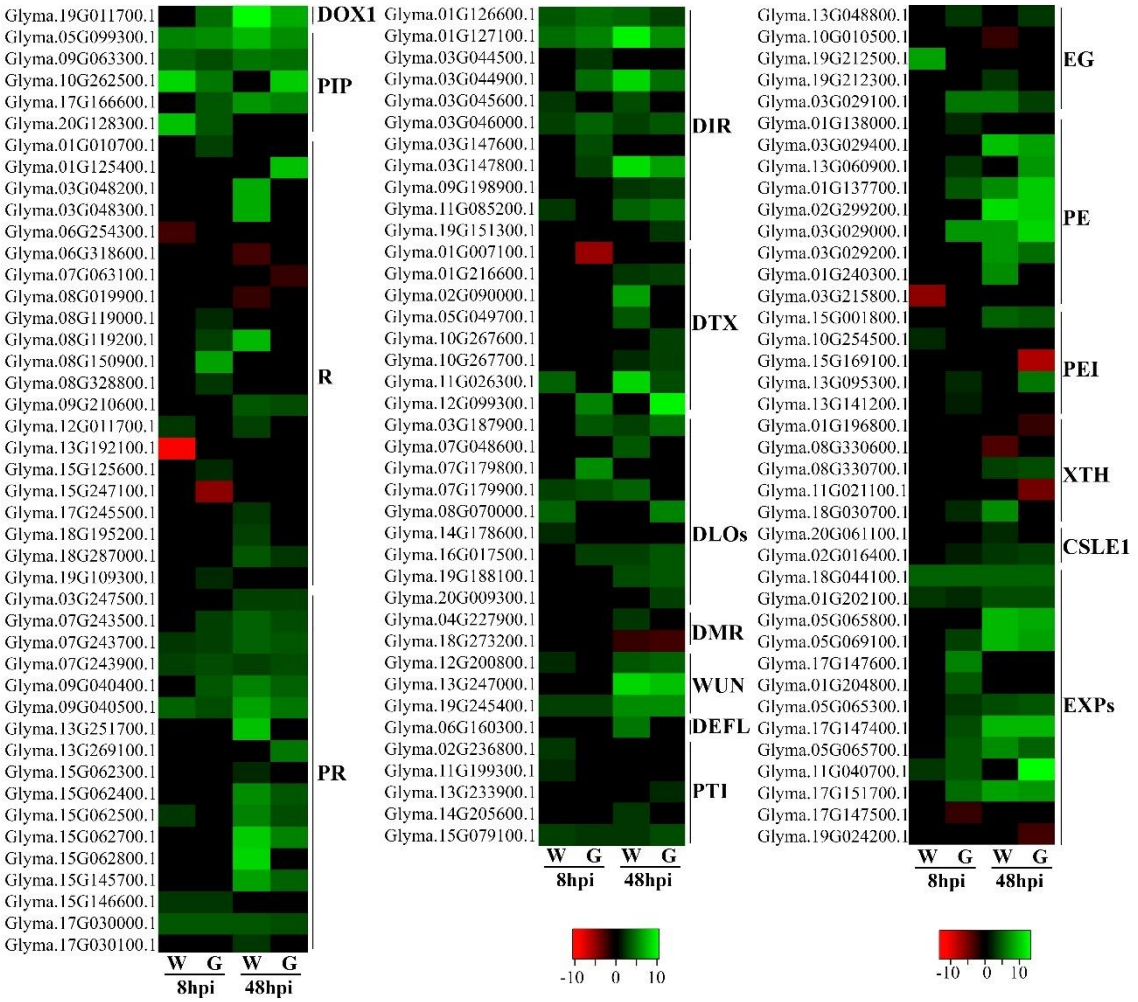


Figure 8 Heatmap of differentially expressed genes (DEGs) encoding for pathogenesis-related (PR) proteins, other defense genes or cell wall modification enzymes. Individual genes are listed and colors represented the log2 fold change value based on the comparison of the transcript levels between *D. caulivora* inoculated and control. Green (upregulated), Red (downregulated), abbreviated as follows: alpha-dioxygenase (DOX1), pathogen-associated molecular patterns-induced secreted protein or peptide (PIP), disease resistance protein (R), pathogenesis-related protein (PR), 11 Dirigent protein (DIR), protein detoxification (DTX), protein DMR6-like oxygenase (DLOs), protein downy mildew resistance (DMR), wound-induced protein (WUN), a defensin-like protein (DEFL), pathogenesis-related genes transcriptional activator (PTI), endoglucanase (EG), pectinesterase (PE), pectinesterase/pectinesterase inhibitor (PEI), xyloglucan endotransglucosylase/hydrolase protein (XTH), cellulose synthase-like protein E1 (CSLE1) and expansins (EXPs).

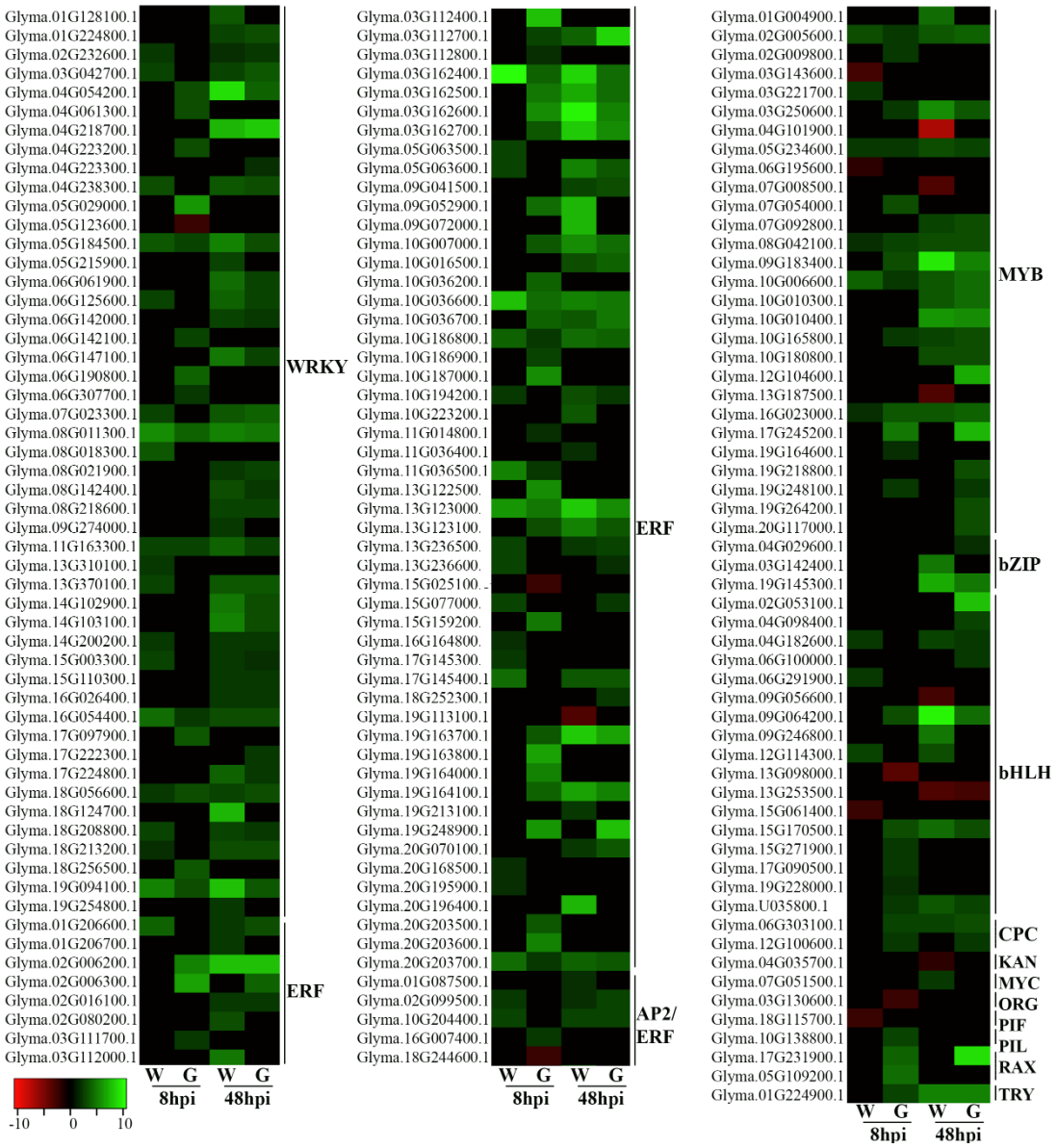


Figure 9 Heatmap of differentially expressed genes (DEGs) encoding for proteins involved in transcription. Individual genes are listed and colors represented the log2 fold change value based on the comparison of the transcript levels between *D. caulivora* inoculated and control. Green (upregulated), Red (downregulated), abbreviated as follows: transcription factors, WRKY transcription factors (WRKY), ethylene responsive transcription factor (ERF), AP2/ethylene responsive transcription factor (AP2/ERF), MYB transcription factor (MYB), bZIP transcription factor (bZIP), bHLH transcription factor (bHLH)

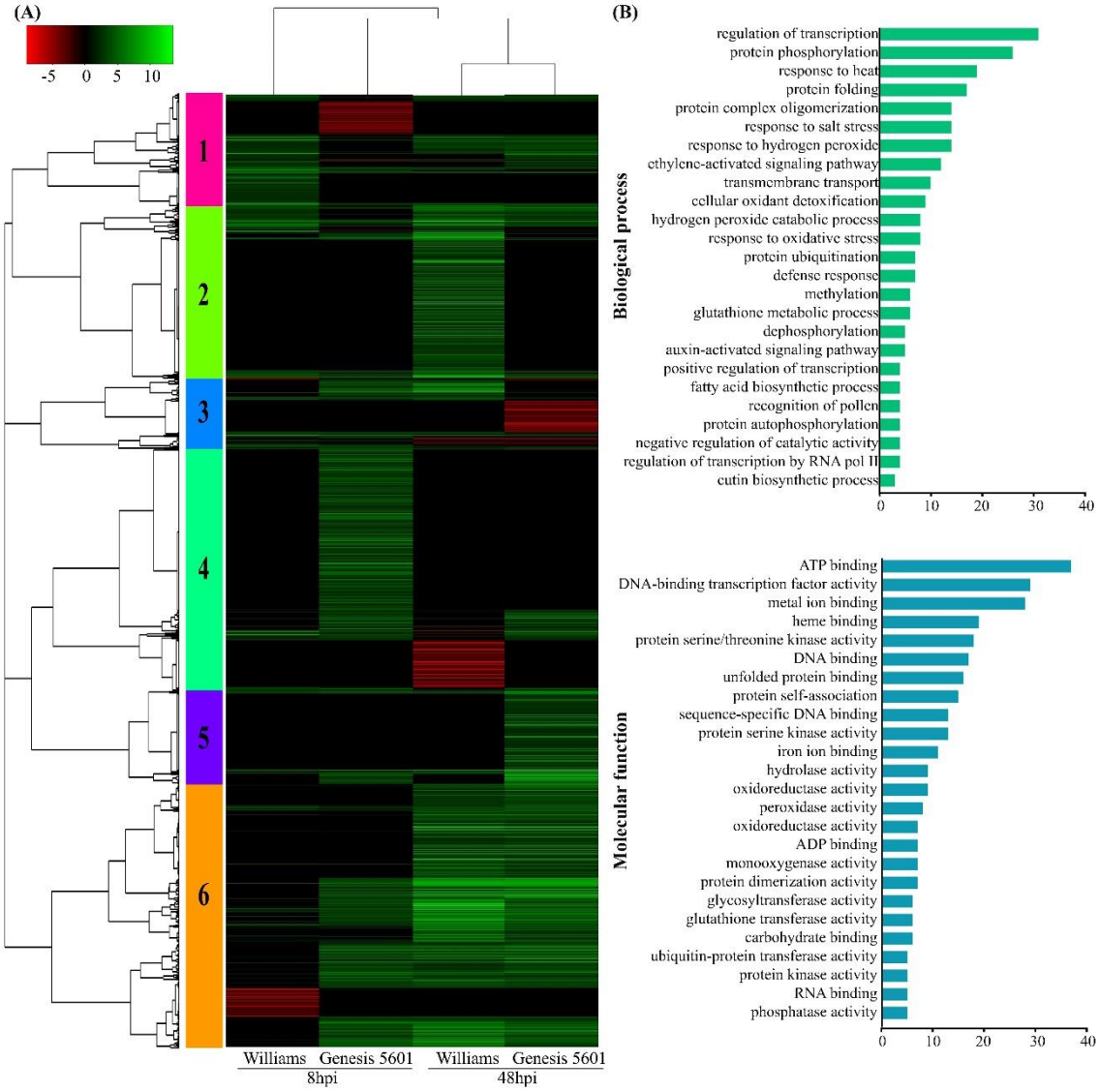


Figure 10 A) Heat map of hierarchical clustering showing of all DEGs in soybean plants inoculated with D. caulivora. Green (upregulated), Red (downregulated). See Table S3 for complete information. **B) Enriched GO terms of the 25 most significantly ($p < 0.05$) of the only upregulated genes in Genesis 5601 at 8 hours post inoculated with *D. caulivora*.**

Supplementary files:

Supplementary Table S1: Summary of mapped reads of the RNA-Seq libraries of soybeans

Supplementary Table S2. List of soybean differentially expressed genes (DEGs)

Supplementary Table S3: Hierarchical clustering of all DEGs in soybean plants inoculated with *D. caulivora*.

Supplementary Table S4: List of DEGs encoding for proteins involved in phenylpropanoid pathways during *D. caulivora* infection in both genotypes at 8 and 48 hpi.

Supplementary Table S5: List of DEGs involved in hormone signaling during *D. caulivora* infection in both genotypes at 8 and 48 hpi.

Supplementary Table S6: List of DEGs encoding for proteins with role in perception and signaling during *D. caulivora* infection in both genotypes at 8 and 48 hpi.

Supplementary Table S7: List of DEGs related to Calcium signal and transporters during *D. caulivora* infection in both genotypes at 8 and 48 hpi.

Supplementary Table S8: List of DEGs encoding for pathogenesis-related (PR) proteins, other defense genes or cell wall modification enzymes during *D. caulivora* infection in both genotypes at 8 and 48 hpi.

Supplementary Table S9: List of DEGs encoding for proteins involved in transcription during *D. caulivora* infection in both genotypes at 8 and 48 hpi.

CONCLUSIONES Y PERSPECTIVAS

Este trabajo realiza aportes originales en la generación de conocimiento básico en el patosistema *D. caulivora-G. max*. El estudio del mismo es importante para comprender las bases moleculares de los mecanismos de resistencia vegetal frente a *D caulivora*.

Los resultados generados sirven de base para avanzar con la investigación, y la información generada podría ser de utilidad en los programas de mejoramiento genético de *G.max* para obtener y seleccionar genotipos resistentes a la enfermedad del CTS para el sector productivo.

En la primera parte del estudio, se identificaron las especies de *Diaporthe* asociadas a lesiones de cancro en Uruguay y se estableció la relación filogenética entre ellas. Se reportó que *D. caulivora* y *D. longicolla* son las especies mayoritarias en el país y que *D. longicolla* puede causar también cancro del tallo de la soja.

Se estableció un método de inoculación de *D. caulivora* en tallos de plantas de soja en condiciones controladas, el cual es reproducible. Además, se confeccionó una escala de evaluación de los síntomas para determinar la severidad de la enfermedad a partir de medidas del largo de las lesiones en el tallo. Esta escala puede ser utilizada por otros grupos de investigación. En el trabajo se describió el proceso de colonización de *D. caulivora* en el tallo de soja mediante la observación al microscopio de cortes histológicos y como complemento se puso a punto una metodología para determinar la biomasa del patógeno en muestras del tallo de soja mediante qPCR.

Se describió los mecanismos de defensa activados en plantas de soja susceptibles ante la infección con *D. caulivora* mediante microscopía y la expresión de genes relacionados con la defensa vegetal. La expresión de genes relacionados con actividades antimicrobianas (PR-2, PR-3, PR-4) y defensinas, lipoxigenasas, peroxidasas, así como genes de la vía de los fenilpropanoides (PAL y CHS), sugieren que estos mecanismos participan en la defensa de las plantas de soja frente a la infección con *D. caulivora*.

La secuenció por primera vez el genoma de *D. caulivora* y se comparó con otros genomas de *Diaporthe* permitió establecer una comparación entre las especies patógenas estudiadas; donde a pesar de tener diferentes hospederos comparten un alto número de genes relacionados con la patogenicidad. Además, se identificaron genes específicos del género *Diaporthe* y otros especie-específicos de *D. caulivora* que podrán ser estudiados con mayor profundidad. Con el análisis del transcriptoma de *D. caulivora* en plantas de soja inoculadas se identificaron un conjunto de genes con expresión diferencial comparado con el transcriptoma de *D. caulivora* en placas de cultivo. El estudio de estos genes reveló que las estrategias de patogenicidad de *D. caulivora* en los primeros estadíos de infección están relacionadas con la degradación y modificación de la pared celular, la detoxificación de compuestos, la actividad de transportadores y la producción de toxinas.

Por último, se identificaron un conjunto de genes con expresión diferencial en plantas de soja inoculadas con *D. caulivora* comparado con plantas sin inocular. Entre las vías más representadas se encontraron la señalización hormonal y la ruta de los fenilpropanoides. La inducción de genes en las vías hormonales es respuesta a *D. caulivora* es interesante y demuestra la importancia de determinar los niveles hormonales, el rol regulatorio sobre otros genes y la interacción entre las hormonas durante la infección.

Los estudios transcriptómicos permitieron identificar además genes que se inducen en las primeras horas de la infección con *D. caulivora* en plantas resistentes y no en plantas susceptibles. Este conjunto de genes incluye varios genes R y receptores involucrados en la percepción del patógeno y constituyen una fuente potencial para aplicaciones biotecnológicas en el mejoramiento del cultivo.

Esta tesis es el primer paso en la caracterización del patosistema *Diaporthe-G. max*. Continuaremos con el estudio de *D. caulivora* en plantas de soja para determinar el mecanismo de infección y las bases moleculares que median la interacción planta-patógeno. Se seguirá analizando en mayor profundidad los resultados transcriptómicos con el objetivo de seleccionar genes candidatos y vías claves que se inducen en la respuesta de soja a la infección con *D. caulivora*, lo cual contribuirá a la generación de conocimiento sobre los mecanismos de defensa involucrados.

Dado los resultados obtenidos y la importancia de las vías hormonales en esta interacción, nos proponemos determinar la concentración de hormonas en plantas de soja a diferentes tiempos de la infección con *D. caulivora.* Esto nos permitirá determinar cuáles hormonas son importantes en la defensa frente a este patógeno. Existen tratamientos con análogos de hormonas vegetales que se utilizan en los cultivos como tratamiento frente a enfermedades como es el caso del 24-epibrasinoesteroide, el etefón (ácido 2-cloroetil fosfónico), 6-bencilaminopurina y auxinas. Además, sería interesante medir los diferentes flavonoides y fenilpropanoides que se sintetizan en plantas de soja en respuesta a la infección con *D. caulivora.* Se van a hacer estudios de microscopía de cortes histológicos de los genotipos de soja estudiados Williams y Genesis 5601 inoculados o no con *D. caulivora,* se van a emplear tinciones de safranina, solofenil-flavin, tolouidina, laminarin (β-1,3 glucano), diaminobencidina y nitro blue tetrazolium para detectar la presencia en el tejido vegetal de compuestos fenólicos, lignina, peróxido de hidrógeno y anión superóxido.

Por último, nos proponemos realizar estudios de infección con *D. longicolla* en plantas de soja en condiciones controladas con el fin de describir los síntomas y el progreso de la enfermedad, así como los mecanismos de defensa involucrados. Será interesante poder comparar los mecanismos de defensa que se activan frente a estos dos patógenos fúngicos causantes de la enfermedad.