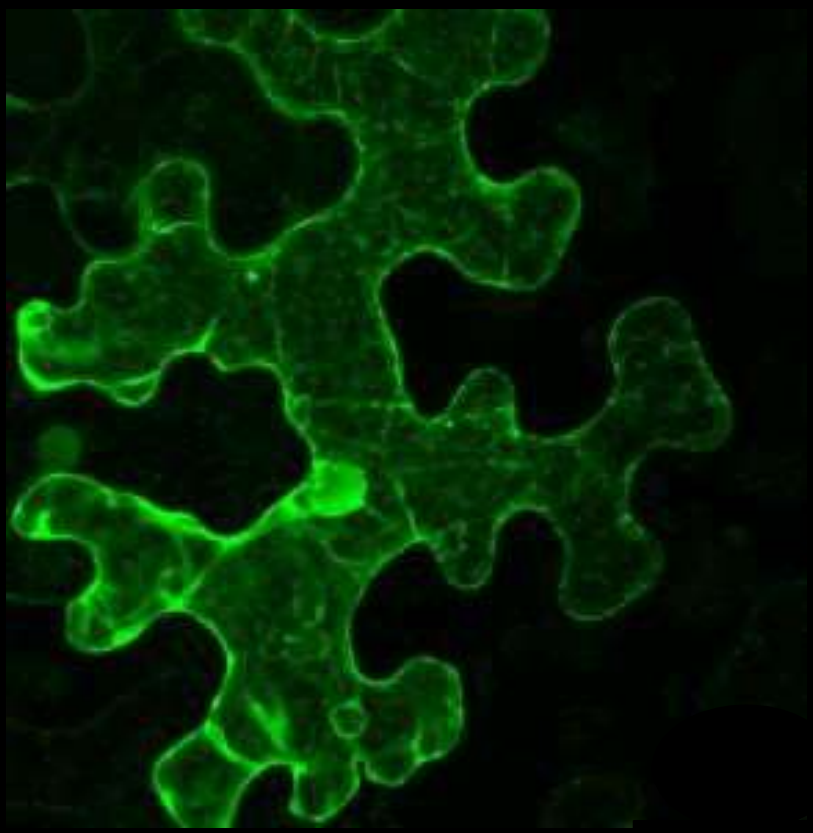
TESIS DOCTORAL

Análisis funcional de dos efectores de *Colletotrichum graminicola*, el agente causal de la antracnosis del maíz.



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Universidad de Salamanca, 2021

Programa de Doctorado en Agrobiotecnología

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Análisis funcional de dos efectores de *Colletotrichum graminicola*, el agente causal de la antracnosis del maíz.

TESIS DOCTORAL

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Dirigido por:

Dr. Michael Ronald Thon

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Salamanca, 2021

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CERTIFICAMOS:

Que la presente memoria titulada "**Análisis funcional de dos efectores de *Colletotrichum graminicola,* el agente causal de la antracnosis del maíz**", ha sido realizada en el Departamento de Microbiología y Genética y en el Instituto de Investigación en Agrobiotecnología (CIALE) de la Universidad de Salamanca, bajo nuestra dirección, por **D. Francisco Borja Cuevas Fernández**, y cumple las condiciones exigidas para optar el grado de Doctor por la Universidad de Salamanca.

Este trabajo se ha llevado a cabo en el Laboratorio 1 del Instituto de Investigación en Agrobiotecnología (CIALE), Departamento de Microbiología y Genética, de la Universidad de Salamanca bajo la dirección de los profesores Dr. Michael Ronald Thon y de la Dra. Serenella Ana Sukno. Durante el desarrollo de la Tesis he disfrutado de una ayuda para contratos predoctorales para la formación de doctores (BES-2016-078373) del Ministerio de Ciencia y Innovación. Dentro de esta ayuda realicé una Estancia Breve que complementó el trabajo presentado en esta Tesis en el laboratorio del Dr. Richard O'Connell, en el centro de investigación "INRAE Laboratory UMR 1290 BIOGER" situado en Grignon, Francia.

Publicaciones científicas relacionadas con los resultados obtenidos en esta Tesis Doctoral y otros trabajos durante el periodo de formación predoctoral:

- **Cuevas-Fernández, F. B**., Robledo-Briones, A. M., Baroncelli, R., Trkulja, V., Thon, M. R., Buhinicek, I., Sukno, S. A. 2019. First report of *Colletotrichum graminicola* causing maize anthracnose in Bosnia and Herzegovina. Plant Disease. Online DOI: https://doi.org/10.1094/PDIS-06-19-1224-PDN.

- **Cuevas-Fernández, F.B**., Sanz-Martín, J.M., Baroncelli, R., Ortiz-Álvarez, J., Robledo-Briones, A.M Dagher, S., Casas Pastor, D.R., Benito, E.P., O'Connell, R.J., Bruno-Barcena, J., Thon, M.R* and Sukno, S.A. Birth by ectopic recombination of a species specific effector that suppresses early host immune response (in preparation for *Plant Cell and Environment*).

- Thon M.R., Baroncelli, R., Crouch. J.A., **Cuevas-Fernández, F.B**, Buhinicek, I., Bettiol, W., Dambolena, J.S., Azcarate Peril, M.A., Malapi-Wight, M., Ortega, V., Betran, J., Tenuta, A., Esker, P, Jackson, T., Hiltbrunner, J. Munkvold, G. Vicente-Villardón, J.L., Grigoriev, I., Sukno, S.A. Population genomics reveals the world-wide phylogeographic structure, migration, and recombination in populations of the maize anthracnose fungus Colletotrichum graminicola (in preparation for *mBio*).

- Robledo-Briones, A.M., **Cuevas-Fernández, F.B**., Baroncelli, R., Thon M.R. and Sukno, S.A. Recent advances in our knowledge of pathogenicity genes in Colletotrichum graminicola. (in preparation for *Plants*).

- Sanz-Martín, J.M., Baroncelli, R., A.M., Dagher, S., Robledo-Briones, A.M., **Cuevas Fernández, F.B.**, Engelsdorf, T., Shoji, J., Revilla-Temiño, P., Vicente-Villardón, J.L., Craven, K.D., Bruno-Barcena, J., Voll, L.M., Thon, M.R. A plant gene acquired by fungi aids in virulence (In preparation for *BMC Biology*).

“La ciencia es más que un simple conjunto de conocimientos, es una manera de pensar”

Carl Sagan.

Dedicado a todos aquellos que tanto me habéis apoyado durante este periodo. Mi más sincera gratitud.

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ABBREVIATIONS AND THERMS OF COMMON USE

3’ UTR	3' untranslated region
5’ UTR	5' untranslated region
ABA	Abscisic acid
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search
bs	Base pairs
cDNA	Complementary DNA
CDS	Coding sequence
CgEP3	*Colletotrichum graminicola* effector protein 3
CgEP6	*Colletotrichum graminicola* effector protein 6
cm	Centimeter
Cº	Celsius grade
Ct	Threshold cycle
CTAB	Hexadecyltrimethylammonium bromide
C-terminal	Carboxy-terminus
DAMP	Damage-associated molecular pattern
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
dpi	Days post-infection
ET	Ethylene
ETI	Effector-triggered immunity
flg22	Flagellin peptide 22
Fw	Forward
GA	Gibberellin
GFP	Green flourescent protein
H2O2	Hydrogen peroxide
hpi.	Hours post-infection
hyg	Hygromycin cassette
ITS	Internal transcribed spacer
Kan	Kanamycin
kb	Kilobases
KCl	Potassium chloride
M	Molar (g/L)
MAFFT	Multiple sequence alignment program
mg	Milligram
min	Minute
mL	Millilitre
MM	Minimum agar
mM	Millimolar (mg/L)
NaCl	Sodium chloride
NLS	Nuclear localization sequence
NP	Nucleoside-phosphorylase
N-terminal	Amino-terminus
OMA	Oatmeal agar
PAMP	Pathogen associated molecular pattern

PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
pH	Potential of hydrogen
PR protein	Pathogenesis-related protein
PRICHEXTENSN	Proline-rich extensins domains
PTI	PAMP-triggered immunity
qRT-PCR	Real-time polymerase chain reaction
QTL	Quantitative trait locus
RNA	Ribonucleic acid
ROS.	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
Rv	Reverse
s / sec	Seconds
SA	Salicylic acid
SNP	Single-nucleotide polymorphism
Δ	Deletion/Delta
µg	Microgram

RESUMEN

RESUMEN

Dentro del grupo de los hongos ascomicetos, se encuentra el género *Colletotrichum* cuyos miembros son capaces de infectar a un gran rango de huéspedes (J. Bailey et al., 1992; D. Prusky et al., 2000). La especie *Colletotrichum graminicola,* causa la antracnosis del maíz cuyos principales síntomas son el tizón foliar y la podredumbre del tallo (Bergstrom & Nicholson, 1999; Jamil & Nicholson, 1991). Esta enfermedad produce grandes pérdidas a nivel global y se estima que en Estados Unidos causa daños valorados por mil millones de dólares al año (R. Dean et al., 2012; T. J. Frey et al., 2011). *C. graminicola* tiene un estilo de vida hemibiotrófico, en el que las primeras etapas de la infección mantienen vivas a las células y se conoce como etapa biotrófica. Posteriormente, cambia a la etapa necrotrófica donde se produce la muerte celular (Bergstrom & Nicholson, 1999; R. J. O'Connell et al., 2012). Para que se dé la interacción compatible con el huésped, el patógeno tiene que vencer su respuesta inmunitaria (J. D. G. Jones & Dangl, 2006). En este sentido, el hongo es capaz de liberar efectores que tienen diversas funciones como, permitir la colonización del huésped, manipular e interferir en la respuesta defensa y proteger al hongo frente a estas (Cook et al., 2015). Los efectores son agrupados dependiendo del lugar donde producen su función como, efectores apoplásticos (liberados en los espacios intercelulares que se forman en el tejido vegetal), efectores citoplasmáticos (transportados al interior celular, en el citoplasma), efectores nucleares (trasladados al interior del núcleo), efectores cloroplásticos (transportados al cloroplasto) y mitocondriales (trasladados a la mitocondria) (Tariqjaveed et al., 2021).

El estudio de los efectores es de vital importancia para tener una mejor comprensión de las interacciones huésped-patógeno. El principal objetivo de este estudio fue analizar funcionalmente dos efectores de *C. graminicola* que podrían estar involucrados en la virulencia del hongo durante la infección del maíz. Así mismo, se realizó la primera descripción de la especie *C. graminicola* en Bosnia y Herzegovina.

En el Capítulo I (***Chapter I***) se procedió a la caracterización del efector *Colletotrichum graminicola* effector protein 3 (CgEP3) y su papel durante la infección del maíz. Esta proteína fue identificada previamente gracias al estudio de Vargas et al. (2016) como un efector putativo que podría tener localización nuclear.

Para comenzar, se llevaron a cabo análisis bioinformáticos para analizar la estructura del gen. En el trabajo de O' Connell et al. (2012) se describió que *CgEP3* era un gen de 446 pares de bases de longitud que constaba de dos exones y un intrón. Sin embargo, durante la realización de experimentos de RT-PCR se obtuvieron amplicones de un tamaño que no se correspondía con el esperado, por lo que se decidió secuenciarlos. Tras un alineamiento tipo MAFFT de las secuencias de los amplicones con *CgEP3,* se demostró que las secuencias tenían homología con el intrón del gen. Por lo tanto, se planteó la hipótesis de que la estructura de *CgEP3* descrita previamente podría estar mal anotada. A continuación, gracias a muestras de RNA-Seq de esporas sin germinar de *C. graminicola* facilitadas por la gentileza del Dr. Stephan Wirsel (Universidad de Martín Lutero de Halle-Weittenberg, Alemania), se determinó la estructura del ARN mensajero putativo de *CgEP3 in silico.* Para confirmar dicha estructura se diseñó un experimento a partir de RT-PCR solapantes que cubrían toda la longitud del locus de *CgEP3.* Como resultado se obtuvo un tránscrito que era más grande que el descrito en la literatura que, además, tenía un intrón en la región no traducida 5'. Posteriormente, mediante la búsqueda de marcos de lectura abiertos en el tránscrito se identificó la región codificante de *CgEP3,* que consistía en un exón de 222 pared de bases.

CgEP3 es un pequeño efector cuya estructura se predijo que tenía un péptido señal de secreción en la región N-terminal que estaba solapado con un dominio del tipo de la superfamilia de las nucleósido-fosforilasas (NP) que posiblemente es parcial. Gracias a los análisis filogenéticos se demostró que *CgEP3* podría haber evolucionado a partir de una duplicación genética que divergió para codificar una proteína efectora. La proteína ancestral podría haber tenido una función nucleósido-fosforilasa. No se encontraron proteínas que fueran similares a CgEP3. Posteriormente se llevaron a cabo análisis de sintenia de la región genómica de *CgEP3.* Se observó que dicha región no tenía similitudes comparada con otras especies del género *Colletotrichum*. Tomando estos resultados en conjunto, se concluyó que CgEP3 era específico de la especie *C. graminicola.* Adicionalmente, comparando la región genómica de *CgEP3* con la especie más emparentada *C. navitas,* se obtuvo que *CgEP3* podría haber sido el resultado de la recombinación ectópica de diferentes partes del genoma. Por otro lado, se procedió a analizar la localización subcelular de CgEP3 mediante análisis *in silico* que predijeron que CgEP3 se trataba de una proteína efectora, secretada y que podría tener localización nuclear. Del mismo modo, a través de la expresión heteróloga y transitoria de *CgEP3* (sin la secuencia del péptido señal predicho) en plantas de tabaco se demostró que la proteína tenía una localización en el citoplasma y el núcleo de las células vegetales. A continuación, se analizó la expresión *CgEP3* mediante experimentos de qRT-PCR (Sanz-Martín, 2016). Estos fueron llevados a cabo a partir de muestras de *C. graminicola* con crecimiento *in vitro* y muestras de hojas de maíz infectadas con el hongo. *CgEP3* se expresaba en esporas sin germinar y durante la etapa biotrófica temprana, teniendo un máximo de expresión a las 12 y 24 horas después de la infección (hpi). Después, los niveles decrecían a partir de las 36 hpi. También se observaron niveles bajos de expresión en el micelio que crecía de manera saprofítica. Gracias a nuevos experimentos realizados con la estirpe de *C. graminicola* p*CgEP3::GFP* (que contenía una construcción del promotor nativo de *CgEP3* fusionado a la región codificante de la proteína fluorescente verde (que fue obtenida previamente, Sanz-Martín, 2016), se corroboró que *CgEP3* se expresaba específicamente en esporas, tubos germinativos y apresorios durante la infección de las hojas de maíz.

Para caracterizar la función de CgEP3, se generaron diferentes estirpes transformantes como: estirpe mutante nula de *CgEP3* (Sanz-Martín, 2016), estirpe de complementación total (que codificaba la secuencia completa de CgEP3), estirpe de complementación parcial (que codificaba una versión truncada de la proteína, solo con el dominio del tipo NP) y la estirpe de expresión constitutiva de *CgEP3.* Una vez generadas las diferentes estirpes transformantes se procedió a su fenotipado. Se analizaron diferentes caracteres del crecimiento y desarrollo de *C. graminicola* mediante cultivos in *vitro.* Como resultados de estos experimentos, no se observaron diferencias en los caracteres analizados, por lo que se sugirió que CgEP3 no afectaba al crecimiento y desarrollo de *C. graminicola.* A su vez, las integraciones de las construcciones genéticas en el genoma de *C. graminicola* no produjeron efectos pleiotrópicos en los caracteres que fueron estudiados.

A continuación, se analizó si CgEP3 estaba implicado en la virulencia de *C. graminicola.* Para ello se infectaron hojas y tallos de maíz. Con respecto a las infecciones de hojas, se observaron diferencias significativas en el tamaño de la lesión de las hojas que estaban infectadas con la estirpe silvestre comparadas con hojas infectadas con la estirpe mutante de *CgEP3,* donde el tamaño de la lesión fue menor. Posteriormente, en las hojas infectadas con la estirpe que expresaba la secuencia de la proteína completa se reestableció el fenotipo inicial, ya que el tamaño de la lesión fue similar al encontrado en hojas infectadas con la estirpe silvestre. Estos resultados demostraron que CgEP3 estaba implicado en la virulencia del hongo. Sin embargo, la

estirpe que expresaba la secuencia de la proteína de manera parcial no recupero el fenotipo de la estirpe silvestre, teniendo un tamaño de la lesión parecido a las hojas infectadas con la estirpe mutante de *CgEP3*. Se sugiere que el hongo podría necesitar la longitud completa de la proteína para reestablecer su función. Adicionalmente, no se contemplaron cambios en el tamaño de lesión de las hojas infectadas con la estirpe que expresaba constitutivamente *CgEP3,* siendo similares a las lesiones producidas por la estirpe silvestre. Este hecho confirmaba que la expresión constante del gen no provocaba un cambio en el fenotipo. Con respecto a las infecciones a 4 dpi, no se observaron diferencias significativas en el tamaño de la lesión de las hojas infectadas entre las estirpes transformantes y la estirpe silvestre. Esto podría deberse a que el hongo ha sido capaz de liberar otros efectores y factores de virulencia que complementan la función de CgEP3. Por último, los resultados obtenidos en las infecciones de tallos a 5 dpi, se correlacionaban con los ya observados en las infecciones de hojas a 3 dpi. Se concluyó finalmente que CgEP3 participaba en la virulencia de *C. graminicola.*

Para comprobar el efecto que causaba la deleción de *CgEP3* durante la infección del maíz, se realizaron diversos ensayos con las estirpes silvestre y mutante de *CgEP3.* En primer lugar, se llevaron a cabo experimentos para cuantificar la biomasa relativa del hongo con respecto a la de la planta durante la infección de hojas de maíz a través de qRT-PCR (Sanz-Martín, 2016). Se demostró que la deleción de *CgEP3* provocaba que *C. graminicola* tuviera una deficiencia en la capacidad de colonización de los tejidos vegetales a 3 dpi ya que se acumulaba menos biomasa. A continuación, se efectuaron experimentos para determinar si la penetración de *C. graminicola* se producía de manera correcta durante la infección de hojas de maíz. Los resultados sugirieron que CgEP3 podría ayudar al hongo durante la penetración de la epidermis de la planta ya que la estirpe mutante penetró un 19% menos que la estirpe silvestre a 36 hpi. Además, la deleción de *CgEP3* produjo un retraso en el desarrollo de la enfermedad (fenómeno que fue observado durante las infecciones de hojas a 3 dpi). Por otro lado, se llevaron a cabo ensayos para comprobar las deposiciones de papilas en la planta, una de las respuestas de defensa que se produce durante los tiempos tempranos de la infección. No se observaron diferencias entre las hojas infectadas con las estirpes silvestre y mutante a 36 hpi, por lo que CgEP3 no afectaba a la deposición de papilas. Posteriormente se hicieron dos ensayos para comprobar si los apresorios podían penetrar correctamente la epidermis de la planta. Gracias al fenómeno de la citorrisis, se estudió la integridad de la pared celular del apresorio. Por otro lado, se analizaron las vesículas lipídicas que se forman dentro de los apresorios. Estas son degradadas para generar compuestos que aumentan la presión interna de los apresorios. Dado que no se observaron diferencias en las curvas de frecuencia de citorrisis entre las estirpes silvestre y mutante, y, además, las vesículas lipídicas del interior del apresorio eran similares, se concluyó que la rigidez de la pared se mantenía en ambas estirpes y la presión interna del apresorio era suficiente para penetrar la epidermis de la planta.

En base a los resultados obtenidos, se concluye que CgEP3 es un efector de pre-penetración específico de la especie *C. graminicola* localizado en el núcleo y citoplasma de la planta. Se ha predicho que CgEP3 es secretado y tiene un dominio del tipo de la superfamilia de las nucleósido-fosforilasas que es parcial. Los análisis filogenéticos demostraron que *CgEP3* podría haber evolucionado a partir de una duplicación genética que divergió para codificar un efector cuya función ancestral fue una nucleósido-fosforilasa. Los análisis de sintenia revelaron que la región genómica de *CgEP3* podría haber evolucionado rápidamente y podría ser el resultado de recombinaciones ectópicas de diferentes partes del genoma. Se propone que CgEP3 juega un papel en la virulencia durante las etapas tempranas de la infección del maíz y podría ayudar al hongo a penetrar la epidermis de la planta. Así, CgEP3 podría ayudar a contrarrestar los

mecanismos de pre-invasión de la planta y así, la colonización podría darse con menos esfuerzo. Este es el primer efector específico de la especie *C. graminicola.*

En el Capítulo II (***Chapter II***) se examinó en mayor profundidad la función de CgEP3 durante la etapa temprana de infección de las hojas de maíz. Para ello, se llevó a cabo un perfil transcripcional para estudiar los genes de maíz que podrían estar inducidos o suprimidos por la proteína efectora CgEP3 a través de análisis de RNA-Seq. De esta forma, se tendría un mayor conocimiento de la respuesta de defensa durante la interacción planta-patógeno y en concreto, por la acción de CgEP3.

Inicialmente, se realizaron infecciones de hojas de maíz con las estirpes silvestre y mutante de *CgEP3.* También se inocularon hojas con agua estéril que fueron usadas como control negativo del experimento. Se recolectaron los puntos de infección a las 24 hpi para su posterior secuenciación mediante Illumina HiSeq 2500. Los datos obtenidos se utilizaron para realizar los análisis de RNA-Seq. Se obtuvo una media de 28 millones de lecturas en las librerías examinadas. Se realizó un control de calidad de las librerías a través de FastQC. Posteriormente las lecturas fueron alineadas con un genoma híbrido compuesto por los genomas del maíz y *C. graminicola* a través de la herramienta HiSat2, cuyo promedio de alineamiento fue del 84%. Se llevaron a cabo análisis de la calidad para comprobar la variabilidad que existía en los tratamientos. Gracias al coeficiente de la correlación de Pearson, se determinó que la correlación era alta para los tratamientos (entre 0,9-1,0 en plantas infectadas con las estirpes silvestre y mutante, y entre un 0,6-0,7 para plantas inoculadas con agua). Del mismo modo, se llevaron a cabo análisis de componentes principales cuyos resultados mostraron que existían variaciones entre los tratamientos, pero no entre las repeticiones biológicas de los tratamientos. Una vez realizadas las comprobaciones, las lecturas fueron mapeadas, se calculó su abundancia y se estimaron los niveles de expresión mediante StringTie. El análisis de los genes diferencialmente expresados (DEGs) se examinó con DESeq2. A continuación, se realizaron comparaciones dos a dos de los tratamientos. Si se comparan los tratamientos de las plantas infectadas con la estirpe silvestre en contra de las plantas infectadas con el control negativo, se obtuvieron 1.916 DEGs a los que se denominaron "genes de respuesta a la enfermedad" y son regulados en presencia del patógeno. Por otro lado, si se enfrentan los tratamientos de las plantas infectadas con la estirpe mutante de *CgEP3* en contra de las plantas infectadas con la estirpe silvestre, se obtuvieron 56 DEGs, a los que se les llamó "genes de respuesta al efector" ya que eran regulados en ausencia de la proteína efectora.

El Capítulo II está basado en la discusión de los genes que eran capaces de responder a CgEP3. De los 56 DEGs, 35 DEGs estaban regulados al alza y 21 DEGs regulados a la baja. Esto significa que los 35 DEGs pueden ser suprimidos, o los 21 DEGs pueden ser inducidos por la acción de CgEP3 cuando las hojas de maíz se encuentran infectadas con la estirpe silvestre. Estos DEGs podrían estar afectados directa o indirectamente por la acción de CgEP3. Sin embargo, se necesitarían análisis experimentales adicionales para contestar a esta cuestión. El bajo número de DEGs sugería que existían pocos genes que estaban respondiendo al efector. Gracias a la herramienta agriGO, se pudo realizar una primera aproximación de las categorías funcionales en las que se verían involucrados estos genes. Las categorías más comunes fueron "procesos metabólicos", "actividad catalítica", "unión" y "procesos celulares". El enriquecimiento en términos de *Gene Ontology* (GO) arrojó que los genes que estaban suprimidos por CgEP3 se relacionaban con la ruta de la biosíntesis de las clorofilas, la ruta de metabolismo de lípidos y el metabolismo secundario a través de la ruta de biosíntesis de terpenoides. Por otro lado, los genes estaban inducidos por CgEP3 estaban enriquecidos en términos de GO que se asociaban

básicamente al metabolismo del azufre. Posteriormente se predijo la localización subcelular de los DEGs a través de diferentes aplicaciones disponibles en línea. Se determinó que once DEGs podrían tener localización nuclear, quince DEGs en el cloroplasto, dos DEGs en el citoplasma y 5 DEGs eran proteínas secretadas. El resto de DEGs tenían una localización ambigua, por lo que no se pudieron agrupar. En conjunto, la localización de estos DEGs en el núcleo u otros compartimentos celulares podrían constituir respuestas aguas abajo de la acción de CgEP3.

Para entender la función biológica que podrían tener los DEGs, se asignaron a categorías funcionales a través de la búsqueda de información disponible en las bases de datos públicas y en la literatura. Se pretendió hacer una revisión de cada uno de los DEGs relacionándolos con las interacciones huésped-patógeno. En cuanto los DEGs que estaban asociados con las proteínas de resistencia a la patogénesis (PR), dos fueron inducidos por CgEP3 (*PRB1-3-like* y *ZmGns*). Estas proteínas son importantes contra las infecciones de los patógenos (Sels et al., 2008). En la categoría de las rutas hormonales se sugiere que la ruta del ácido salicílico (SA) podría estar activada debido a la inducción de las proteínas PR. También se indujeron dos DEGs, uno de los cuales estaba relacionado con la ruta de señalización del etileno (*EIN2-like*), y otro con la ruta de biosíntesis de las giberelinas (*CYP P450*). Por otro lado, CgEP3 suprimió la acción de un DEG que estaba implicado en la ruta de biosíntesis de ácido abscísico (ABA) (*NCED8*). Se sabe poco del mecanismo de respuesta hormonal del maíz contra *C. graminicola.* Se ha visto que hormonas como el SA y ABA podrían tener un papel importante en la respuesta de defensa del maíz (Vargas et al., 2012; Balmer et al., 2013). En la categoría de factores de transcripción se localizaron dos DEGs que eran suprimidos por CgEP3 (*ZmMYBRE115* y *ZmCOL16*), mientras que tres eran inducidos por la acción del efector (*ZmWRKY83, ZmTPL6* y *GT*). Los factores de transcripción controlan la expresión de genes que son importantes contra estreses bióticos (Ng et al., 2018). En la categoría de DEGs asociados con la fotosíntesis aparecieron genes que participaban en la síntesis de clorofilas y carotenoides (*DXS1, CHLD, ZmPORA*) que estaban suprimidos por CgEP3; DEGs que se relacionaban con los fotosistemas y la cadena de transporte electrónico (*ZmLHCBa, LHCA* y *NdhB*) que estaban suprimidos por la proteína efectora y un DEG (*DNA translocase*) que estaba inducido por el efector. Debido al ataque de los patógenos, se incrementa los requerimientos fotosintéticos en el huésped. La fotosíntesis produce energía y moléculas precursoras que son necesarias para la respuesta de defensa (Hammerschmidt 1999; Swarbrick et al., 2006). En la categoría de DEGs vinculados al metabolismo primario, CgEP3 afectó a numerosas rutas: metabolismo del azufre (*ZmSULTR1.2b* y *ATPS,* que estaban inducidos), metabolismo del selenio (*SELENBP1*, que era suprimido), metabolismo del carbono (*TPI* y *ENO* que se encontraba suprimido), metabolismo de lípidos (*SMO1*, que era suprimido) y metabolismo del hierro (*DMSA1* y *BOLA1,* que estaban inducidos). Se ha visto como el metabolismo primario de la planta controla diferentes cascadas de transducción de señales que permiten la respuesta de defensa de la planta (Rojas et al., 2014). En la categoría del metabolismo secundario se obtuvo que había tres DEGs asociados a la ruta de los fenilpropanoides (*PKS1, ATD* y *OMT*) que eran suprimidos por CgEP3, mientras que un DEG era inducido por el efector (*TPS6*). Los metabolitos secundarios producidos por las plantas pueden ayudar en la respuesta de defensa (Hartmann, 2007). La ruta de los fenilpropanoides es una de las más importantes implicadas en este propósito (Pan et al., 2012). En la categoría de genes relacionados con el metabolismo del ARN, se encontraron cuatro DEGs que estaban suprimidos por CgEP3 (*SR, PPR310, mS34* y *RRL10A*). Las proteínas que interaccionan con el RNA participan en numerosos procesos como la respuesta de defensa de la planta (Lorković, 2009). En la categoría de plegamiento de proteínas y degradación aparecieron dos DEGs que codificaban proteínas con dominios U-box y un DEG (*CYP*), todos ellos suprimidos por CgEP3, además de otro

DEG que estaba inducido por el efector (*DNAJC9*). La actividad de las proteínas puede ser modulada por modificaciones postraduccionales para dar estabilidad o permitir su degradación. Estos procesos son importantes contra estreses bióticos (Zeng et al., 2006). En la categoría de DEGs vinculados a la remodelación de la pared celular, se encontró un DEG (*GT47*), que estaba suprimido por el efector. La estructura y la composición de las paredes celulares se encuentran en constante cambio frente a diferentes respuestas y procesos biológicos. La integridad de la pared celular ayuda en las interacciones con el ambiente y contra patógenos (Gigli-Bisceglia et al., 2020). Por otro lado, se localizaron otros DEGs que no se fue capaz de asignar a una categoría funcional que estaban suprimidos (*CK1, SEY1, DEH* y *Phytepsin*), o inducidos (*APY, dehydratation-responsive, GLP3* y *MOI1*) por CgEP3. Del mismo modo, se identificaron nueve DEGs que no fue posible realizar una anotación (seis estaban suprimidos por CgEP3 y tres eran inducidos). Estos podrían representar nuevos mecanismos de defensa de la planta. Por último, aunque no se encontraron DEGs relacionados directamente con el estallido de especies reactivas de oxígeno (ROS), se identificaron algunos que podrían estar asociados indirectamente (*TPI, BOLA1, GLP3* y *SELENBP1*). Durante el ataque de los patógenos de plantas, una de las primeras respuestas es la acumulación de ROS en los puntos de infección que permite activar la respuesta de defensa (Nürnberger et al., 2004).

Tomando estos resultados en conjunto, se describieron 56 DEGs que eran capaces de responder al efector. Del conjunto de los DEGs se diferenciaron dos grupos, 35 DEGs y 21 DEGs que eran suprimidos o inducidos por la acción de CgEP3 respectivamente, durante la infección de hojas de maíz con la estirpe silvestre de *C. graminicola* a las 24 hpi. CgEP3 podría contrarrestar las respuestas de defensa del maíz a través de los DEGs que eran suprimidos. Por otro lado, CgEP3 podría ser reconocido por la planta y, en consecuencia, activar genes que estimulaban la respuesta inmunitaria. Estos se correspondían con los DEGs que eran inducidos por el efector. Se concluye que estos grupos de genes representan mecanismos de defensa independientes que pueden ser suprimidos o inducidos por CgEP3 durante la etapa temprana de la infección de las hojas del maíz.

En el Capítulo III (***Chapter III***) se realizó un análisis funcional del efector *Colletotrichum graminicola* effector protein 6 (CgEP6) para conocer el papel que podría jugar durante la infección del maíz. Gracias al trabajo de Vargas et al. (2016) se identificó esta proteína que podría actuar como un efector putativo y está localizado en el núcleo de las células vegetales del huésped.

Para comenzar se realizaron análisis bioinformáticos para analizar la estructura de la proteína. Se obtuvo que CgEP6 tenía un péptido señal en la región N-terminal y cuatro dominios ricos en extensinas (PRICHEXTENS) en su estructura. Del mismo modo, se predijo que CgEP6 tenía una secuencia de localización nuclear en la región C-terminal. Se observó un alto contenido de prolinas en su secuencia (22%). Además, basados en estudios de humanos se describieron cinco motivos "poliprolina", tres de los cuales tenían seis o más prolinas consecutivas que podrían interaccionar con el ADN/ARN. Posteriormente se realizaron análisis filogenéticos para examinar cómo había evolucionado CgEP6. Gracias a la herramienta HMMER, se encontraron 21 proteínas que eran homólogas a CgEP6 en diferentes especies del género *Colletotrichum.* Además, estas proteínas tenían una única copia en el genoma. No se encontraron proteínas homólogas en otras especies fuera de este género, por lo que CgEP6 es un efector que se encuentra conservado en el género *Colletotrichum.* Un alineamiento de tipo MAFFT demostró que todas las proteínas tenían un péptido señal en la región N-terminal y eran ricas en prolinas. Además, dos especies (*C. sublineola* y *C. eremochloae*) presentaron dominios PRICHEXTENSN similares a *C.*

graminicola. Posteriormente, se construyó un árbol filogenético con el que se determinó que CgEP6 y sus homólogos se distribuían en siete de los complejos de especies descritos: orbiculare, gloeosporioides, acutatum, destructivum, sphaetianum, graminicola y caudatum. A continuación, se realizaron análisis de sintenia de la región genómica de *CgEP6* cuyos resultados mostraron que esta se conservaba parcialmente en el género *Colletotrichum* en los complejos acutatum, destructivum, sphaetianum, graminicola y caudatum. La estructura génica de *CgEP6* (dos exones y un intrón) se conservó en todas las especies analizadas salvo en tres (*C. caudatum*, *C. salicis*, y *C. fructicola*). Aguas arriba del gen *CgEP6,* se encontró una región de 7,3 kb que podría haber surgido a partir de los reordenamientos genéticos producidos en el genoma ya que esta no tenía similitud con el resto de las especies. Después se estudió la evolución de *CgEP6* en doce estirpes de *C. graminicola.* Estudios previos con siete estirpes revelaron que la región codificante de este gen estaba sometida a presión de selección positiva (Rech et al., 2013). Gracias al análisis de las substituciones que se habían producido en la región codificante del gen en las doce estirpes, permitió identificar cuatro alelos diferentes de CgEP6 que variaban en tamaño y contenido de prolinas.

Una vez examinada la evolución de CgEP6, se procedió a estudiar su localización subcelular a través de análisis *in silico* y experimentales. Los análisis *in sílico* demostraron que CgEP6 era un efector, secretado, que tenía una secuencia de localización nuclear en su extremo C-terminal y que se localizaba en el núcleo. Para confirmar esta hipótesis se llevaron a cabo experimentos de expresión de manera heteróloga y transitoria de *CgEP6* en plantas de tabaco. La secuencia del gen no contenía el péptido señal predicho. Los resultados mostraron que CgEP6 estaba localizada en el citoplasma y núcleo de las células vegetales. Posteriormente, a través de experimentos de qRT-PCR fue examinada la expresión del gen *CgEP6.* Para ello se utilizaron muestras del hongo creciendo *in vitro* y también muestras de hojas de maíz infectadas con *C. graminicola* en diferentes tiempos. *CgEP6* se expresó durante la etapa biotrófica de la infección teniendo un pico de expresión entre las 12 y 24 hpi. Después, la expresión decrecía rápidamente, aunque fue apreciable a las 36 y 48 hpi. Los valores de expresión se mantuvieron bajos en los demás tiempos, incluidos aquellos en los que el micelio estaba creciendo *in vitro* (esporas sin germinar y micelio creciendo saprofíticamente). Este hecho podría ser una consecuencia del desarrollo de la infección de *C. graminicola* en el maíz ya que biotrofía y necrotrofía conviven simultáneamente. Mientras que las hifas necrotróficas se desarrollan en el centro de la colonia, las hifas biotróficas continúan creciendo en los bordes del punto de infección (Crouch et al., 2014).

Para conocer la función de CgEP6 en mayor profundidad, se procedió a su caracterización funcional. Para ello, se generó la estirpe mutante nula de *CgEP6* a través del método de doble unión. Los mutantes fueron comprobados por PCR. Tras analizar un gran número de transformantes putativos, solo se encontró una estirpe en que la recombinación homóloga se produjo de manera correcta. Por lo tanto, la tasa de este tipo de recombinación fue muy baja, 1,4%. A continuación, se procedió al fenotipado de la estirpe mutante de *CgEP6* a través del cultivo *in vitro* del hongo en diferentes medios. Se observó un retardo en la tasa de crecimiento de la estirpe mutante comparada con la estirpe silvestre en medio PDA. Posteriormente, se suplementó el medio PDA con diferentes compuestos que ocasionaban situaciones de estrés (salino, oxidativo y de la pared celular del hongo). No se encontraron diferencias para los estreses salino y oxidativo. En cambio, se observó un retraso del crecimiento de la estirpe mutante de *CgEP6* comparado con la estirpe silvestre en el medio que producía un estrés en la pared celular del hongo (PDA suplementado con rojo Congo). Para comprobar la sensibilidad del hongo frente al rojo Congo, se calculó el porcentaje de tasa inhibitoria. Comparado con la estirpe

silvestre, la estirpe mutante mostraba un valor 1,63 veces mayor del porcentaje de tasa inhibitoria, por lo que esta estirpe era mucho más sensible al rojo Congo que la estirpe silvestre. Por lo tanto, se sugirió que la integridad de la pared celular en la estirpe mutante de *CgEP6* estaba afectada. Para terminar, se realizaron infecciones de hojas de maíz para conocer si CgEP6 estaba implicado en la virulencia de *C. graminicola.* En este sentido se infectaron hojas con las estirpes silvestre y mutante de *CgEP6* y se comprobaron los resultados a 3 dpi y 4 dpi. En ambos tiempos, se observó que el tamaño de la lesión en hojas infectadas con la estirpe mutante de *CgEP6* fue menor comparado con las hojas que estaban infectadas con la estirpe silvestre. El tamaño de la lesión se redujo en un 25,0% a 3 dpi y un 27,9% a 4 dpi. Se concluyó finalmente que CgEP6 participaba en la virulencia de *C. graminicola* durante el desarrollo de la antracnosis en el maíz.

Tomando todos estos resultados en conjunto, se concluye que CgEP6 es un efector que se conserva en el género *Colletotrichum* que se encuentra localizado en el núcleo y el citoplasma de la planta. CgEP6 es rico en prolinas y tiene en su estructura cuatro dominios PRICHEXTENSN y cinco motivos "poliprolina". Basados en estudios previos, *CgEP6* podría estar sujeto a presión de selección lo que habría posibilitado la aparición de cuatro alelos diferentes de CgEP6 durante la evolución. *CgEP6* se expresa específicamente en la etapa biotrófica temprana durante las infecciones de hojas de maíz. CgEP6 participa en la virulencia de *C. graminicola* durante la infección de hojas y también puede tener un papel en la integridad de la pared celular del hongo.

En el Capítulo IV (***Chapter IV***) se expone la publicación de la primera descripción de *C. graminicola* en Bosnia y Herzegovina (Cuevas-Fernández et al., 2019). Se recibieron muestras de tallos de maíz que mostraban síntomas de la antracnosis (pudrición del tallo) de campos de una zona del noreste de Bosnia y Herzegovina (Odžak, Posavka Mahala). Se aislaron pequeños trozos de tejido del maíz que se crecieron *in vitro* de los que se aislaron diferentes estirpes de hongos. Tras analizar su fenotipo, algunas de ellas mostraban similitudes con los caracteres morfológicos de la especie *C. graminicola* (micelio de color gris oscuro, masas de esporas de color naranja y esporas con forma fusiforme). Se realizaron cultivos monospóricos de las estirpes de las que se extrajo ADN genómico. Para el genotipado de las estirpes se realizó una PCR de la región del espaciador transcrito interno (ITS), cuyos amplicones fueron secuenciados. Tras una búsqueda BLASTN de las secuencia ITS, mostraban un 100% de similitud con la región ITS de *C. graminicola.* Por último, para verificar los postulados de Koch, se realizaron infecciones de hojas con dos de las estirpes aisladas de Bosnia y Herzegovina. Tras 6 dpi, se observaron los síntomas típicos del tizón foliar del maíz (lesiones de color marron necróticas, donde los márgenes son cloróticos). Además, se analizaron las lesiones en el miscroscopio que mostraban acérvulos, setas y conidiosporas. Finalmente se concluyó que la especie aislada de los campos de maíz de Bosnia y Herzegovina se trataba de la especie *C. graminicola.*

INTRODUCCIÓN

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El reino de los hongos está compuesto por organismos eucariotas que puede comprender 120.000 de especies descritas (Hawksworth & Lücking, 2017) . Es un grupo de organismos muy variado que tiene diferentes estilos de vida. Pueden interaccionar con numerosos organismos a través del mutualismo, parasitismo y comensalismo. Los hongos se encuentran distribuidos en todo el planeta y causan una gran impacto como descomponedores o (J. J. Choi & Kim, 2017; Cracraft & Donoghue, 2004; Petersen, 2013; Stajich et al., 2009). Algunas de estas especies son patógenas de plantas y pueden colonizar diferentes pueden colonizar diferentes especies de cultivo, bosques, plantas ornamentales y de los ecosistemas naturales (Willis, 2018). Los hongos fitopatógenos a menudo se asocian a dos grandes filos, Ascomycota y Basidiomycota. El filo Ascomycota se encuentra representado por clases como, Sordariomycetes (que incluyen los géneros *Colletotrichum* o *Magnaporthe*), Leotimycetes (con el género *Botrytis*) o Dothideomycetes, (que incorpora el género *Cladosporium*). Por otra parte, en el filo Basidiomycota aparecen dos grandes grupos de patógenos de plantas que producen las rollas y los tizones, que se encuentran dentro de los Puccioniomycetes y dentro del subfilo Ustilaginomycotina, respectivamente (Doehlemann et al., 2017).

Los hongos patógenos pueden clasificarse en dos grupos principales dependiendo de su estilo de vida. Los hongos biotróficos son aquellos que se encuentran relacionados de manera íntima con la planta y usan los tejidos vivos para nutrirse. Estos mantienen a las células con vida durante todo su ciclo (Lewis, 1973). Son capaces de liberar efectores que ayudan en dicho mantenimiento, además de suprimir la respuesta de defensa de la planta (Doehlemann et al., 2017; Kemen et al., 2005, 2013; Petre et al., 2016; Petre & Kamoun, 2014). Los hongos biotróficos forman una estructura que utiliza para nutrirse, el haustorio, que se ubica bajo la pared celular de la célula vegetal que no perturba la membrana celular (Mendgen and Hahn, 2002; Staples, 2001). Se encuentran especies como *Ustilago maydis, Blumeria graminis, Melampsora larici-populina* y *Puccinia graminis* (de Guillen et al., 2019; Doehlemann et al., 2017; Duplessis et al., 2011; Kämper et al., 2006; Spanu et al., 2010). Los hongos necrotróficos, son aquellos que aniquilan los tejidos del huésped para obtener nutrientes y para ello, utiliza una gran cantidad de enzimas hidrolíticas y toxinas (Lewis, 1973). Se incluyen especies como *Botrytis cinerea, Cochliobolus heterostrophus* o *Pyrenosphora tritici-repentis* (Bolton et al., 2006; Faris et al., 2010; Wolpert et al., 2002). Además, se encuentra un tercer grupo que son los hongos hemibiotróficos, que comienzan con una etapa inicial biotrófica y posteriormente cambian a una segunda etapa, la necrotrófica (J. Bailey et al., 1992; Luttrell, 1974; Parbery, 1996). La fase biotrófica de la infección puede ser variable en el tiempo dependiendo de la especie y las condiciones ambientales (O'Connell et al., 2012; Perfect et al., 1998; Yi & Valent, 2013). Al igual que los hongos biotróficos, son capaces de liberar efectores que ayudan a inhibir la respuesta inmune de la planta (Hemetsberger et al., 2012; Kleemann et al., 2012; Koeck et al., 2011; Saitoh et al., 2012). Tras la penetración de la epidermis de la planta, producen unas hifas especializadas que invaginan la membrana plasmática de las células vegetales (Perfect & Green, 2001; Vargas et al., 2012). Posteriormente el hongo cambia su ciclo a la etapa necrotrófica y desarrolla otro tipo de hifas que liberan toxinas y enzimas que producen la muerte celular (Amselem et al., 2011; Horbach et al., 2011). Dentro de este grupo de hongos se encuentran especies de los géneros *Magnaporthe, Colletotrichum, Fusarium, Verticillium,* entre otros (Fradim & Thomma, 2006; L.-J. Ma et al., 2013; Ploetz, 2015; Yi & Valent, 2013). Esta clasificación permite realizar una clasificación básica de los estilos de vida que son comunes en estos organismos, sin embargo, la relación no es tan estricta entre los grupos (Oliver & Ipcho, 2004).

Los hongos patógenos de plantas suponen una gran amenaza para la agricultura. Aunque muchas de las enfermedades pueden ser controladas, gran parte de los hongos causan epidemias asoladoras que provoca numerosas pérdidas en la productividad de los cultivos (Fisher et al., 2012; Olsen et al., 2011; Pennisi, 2010).

Existen numerosos factores que permiten que aumente el impacto de los hongos patógenos a nivel mundial como su transporte (Smith & Read, 2008; Willis, 2018), la plantación de cultivos de un solo tipo(Averill et al., 2014; Clemmensen et al., 2013; Willis, 2018) y el cambio climático, que favorecen que los patógenos puedan ocupar nuevas regiones (van der Heijden et al., 2015; Willis, 2018). Gracias a la secuenciación masiva de genes, el número de genomas de hongos ha aumentado a gran velocidad en los últimos años (Jumpponen & Trappe, 1998; Mastouri et al., 2010; Shoresh et al., 2010; Willis, 2018). Su estudio nos permite tener una mejor comprensión de las interacciones huésped-patógeno y en concreto, de las enfermedades que producen los patógenos para poder combatirlas de una manera más eficaz disminuyendo su impacto en la seguridad alimentaria y medio ambiente (Keith & Jenny, 1999; Kernaghan, 2005; van der Heijden et al., 1998; Willis, 2018).

Para el desarrollo de programas eficaces y preventivos de la propagación de enfermedades de hongos, se deben realizar: estudios epidemiológicos y de genética de poblaciones; evaluaciones de riesgo y vigilancia; y seguimiento de las condiciones climáticas. Existen diferentes formas para controlar la diseminación de los hongos como la reducción en el transporte de plantas, exclusión de aquellas que están contaminadas, la mejora del diagnóstico de enfermedades y el desarrollo de fungicidas específicos (Gange et al., 2012; Willis, 2018).

Colletotrichum, un género de hongos patógenos de plantas

El género *Colletotrichum* Corda (1831) pertenece a la familia *Glomerellaceae* (Glomellales, Sordariomycetes) (Bhunjun et al., 2021; Hyde, 2020; Hyde et al., 2019; Maharachchikumbura et al., 2016). Está formado por especies de hongos con diferentes estilos de vida que van desde patógenos de plantas, causando la enfermedad de la antracnosis, hasta especies endofíticas y saprofíticas (Bhunjun et al., 2021; Talhinhas & Baroncelli, 2021). Como patógenos, se encuentran distribuidos por todo el globo y ocasionan dicha enfermedad en un gran número de huéspedes diferentes: en plantas dicotiledóneas (como fresa, manzana, cítricos y frutos con hueso), monocotiledóneas (cereales como el maíz y el sorgo), además de infectar helechos y pinos (Talhinhas & Baroncelli, 2021). Algunas especies de *Colletotrichum,* son capaces de interaccionar con un solo huésped, mientras que otras pueden realizarlo con numerosos huéspedes, lo que complica el control de la antracnosis (Bhunjun et al., 2021; da Silva et al., 2020).

Los principales síntomas de la enfermedad son la aparición de lesiones necróticas con forma ovalada o angulosa (Nicholson and Warren, 1976; Bergstrom et al., 1999; Talhinhas & Baroncelli, 2021). Además, tras el cosechado de las frutas pueden producir la podredumbre de estas. Se ha visto a su vez que también pueden infectar hojas, tallos, tubérculos y plántulas de los cultivos (Crouch & Beirn, 2009; Green & Simonds, 1994; Thomas & Sweetingham, 2004) **(Figura 1)**. Todo ello supone que la antracnosis ocasione numerosas pérdidas, sobre todo en cultivos de gran importancia a nivel global (Cannon et al., 2012; D. Prusky et al., 2000; Talhinhas et al., 2005). El género *Colletotrichum* está considerado como uno de los diez grupos de hongos más patogénicos de plantas (Brundrett & Tedersoo, 2018; Willis, 2018; Willis & McElwain, 2013).

Figura 1. Principales síntomas de la antracnosis ocasionados por *Colletotrichum* spp., en diferentes huéspedes. A) adhatoda de Ceilan (*Adhatoda vasica*). **B)** *Pedilanthus tithymaloides*. **C)** *Aloe vera*. **D)** *Achyranthes aspera*. **E)** vid (*Vitis vinifera*). **F)** Caqui (*Diospyros kaki*). **G)** Pimiento (*Capsicum* sp.). **H)** Banano (*Musa* sp.). **I)** *Sansevieria trifasciata.* **J)** Cinia (*Zinnia* sp.). **K)** Pimiento (*Capsicum* sp.). **L)** *Boerhavia diffusa*. **M)** Bananas. Esta figura ha sido extraída del estudio de Gautam, (2014).

El género *Colletotrichum* comprende un total de 257 especies. Basándose en análisis filogenéticos, estas especies se encuentran asociados en 15 complejos de especies: orbiculare, dracaenopilum, manum, orchidearum, truncatum, agaves, boninense, gloeosporioides, gigasporum, dematium, destructivum, sphaetianum, graminicola, caudatum y acutatum (Talhinhas & Baroncelli, 2021) **(Figura 2)**.

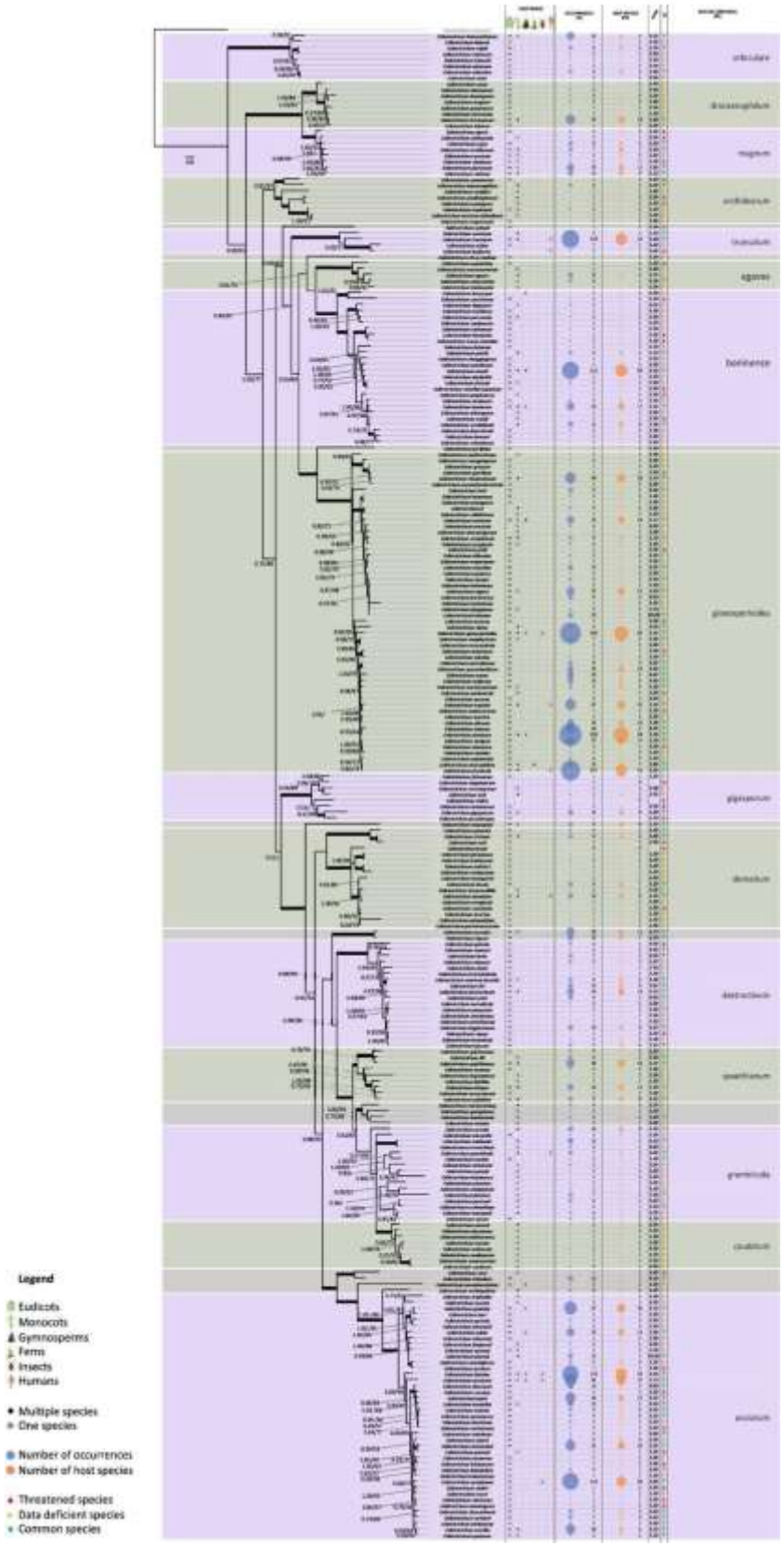


Figura 2. Árbol filogenético que representa las relaciones entre las especies del género *Colletotrichum.* Se representan 15 complejos en los que se encuentran agrupadas las especies. También se incluye el huésped donde se desarrollan, si son específicos de un huésped o de múltiples, el número reportes que existen sobre la especie (O) y el huésped (H), ratio O/HS y el si las especies dentro del género se encuentran amenazadas, son comunes o sin datos suficientes. Esta figura ha sido extraída del estudio de (Talhinhas & Baroncelli, 2021).

Las especies que se encuentran dentro de cada complejo muestran un vínculo cercano entre ellas y comparten el método de infección y de colonización de los huéspedes (Damm et al., 2012a; Damm, et al., 2012b; Silva et al., 2017; Jayawardena et al., 2016; Sanders & Korsten, 2003). Las especies del género *Colletotrichum* se estima que divergieron entre los 32,2 y 4,8 millones de años durante la época del Oligoceno (periodo Paleógeno de la era Cenozoica). Este periodo pudo calcularse gracias a diferentes análisis genómicos basados en la filogenia, evolución y métodos relacionados con la cohesión o coalescencia (Bhunjun et al., 2021; U Damm et al., 2019; Jayawardena et al., 2016; Marin-Felix et al., 2017).

El género *Colletotrichum* comprende especies con distintos estilos de vida

Con respecto a los estilos de vida de las especies del género *Colletotrichum,* muestra una gran diversidad entre los grupos (Bailey & Jeger, 1978; Perfect et al., 1999; Prusky et al., 2013; Rodriguez & Redman, 2008; Vargas et al., 2012), y se incluyen los estilos de vida necrotrófico, hemibiotrófico, latencia o quiescencia y endofítico. Aunque el estilo de vida hemibiotrófico es el que se suele dar con mayor asiduidad (Auyong et al., 2012; Barimani et al., 2013; De Silva et al., 2017; Münch et al., 2008; Peres et al., 2005; Perfect et al., 1999). Todavía hoy se desconocen los mecanismos genéticos y bioquímicos por los que se regulan los diferentes estilos de vida. Probablemente se deba a una predisposición genética, o bien sea el resultado de la respuesta fisiológica frente a diferentes factores del ambiente tanto bióticos, como abióticos, o de ambos (Kabbage et al., 2015; Redman et al., 2001; Vargas et al., 2012). Se describirán de manera breve cada uno de los estilos de vida (Bailey & Jeger, 1978; Perfect et al., 1999; Rodriguez & Redman, 2008):

1)Necrotrofía. Pertenecen a este grupo aquellas especies que se desarrollan sobre el huésped produciendo la muerte celular. Son capaces de liberar enzimas y toxinas que le ayudan a producir la muerte de los tejidos del huésped. El patógeno es capaz de alimentarse de los componentes que son liberados tras la muerte celular y los utiliza para acabar su ciclo (Lewis, 1973; Schäfer, 1994; Walton, 1996). Además, algunas especies endofíticas mantienen el estilo de vida necrotrófico durante todo su ciclo (Crouch & Beirn, 2009; Manamgoda et al., 2013; Nicholson & Warren, 1981).

2)Latencia o quiescencia. Son aquellas especies que necesitan de un estado de dormancia durante su ciclo de vida, que suele ser prolongado. En esta etapa el crecimiento del patógeno está detenido, por lo que se actividad es muy reducida. Posteriormente cambian a la fase activa del ciclo (Dov Prusky et al., 2013). Se suele dar en especies de *Colletotrichum* que ocasionan enfermedades en los frutos tras su cosechado como *C. gloeosporioides* en el aguacate o *C. acutatum* en la almendra (Adaskaveg & Förster, 2000; Prusky, 1996).

3)Endofistismo. Se encuentran especies de *Colletotrichum* que son simbiontes y que viven en el interior del huésped y que no produce una enfermedad (Hardoim et al., 2015; Redman et al., 2001; Rodriguez et al., 2005; Vieira et al., 2014; Wilson, 1995). Un gran número de las especies endofíticas se encuentran asociadas a los complejos gloeosporioides, graminicola y boniense (Damm et al., 2012b; Gaudelli et al., 2017; Vieira et al., 2014; Weir et al., 2012).

4)Hemibiotrofía. Muchas de las especies del género muestran este estilo de vida, por lo que se explicará más detalladamente. En general, el ciclo empieza cuando una espora germina y desarrolla una estructura especializada que se denomina apresorio. Gracias a esta estructura, la cutícula de la planta y las células epidérmicas pueden ser penetradas por el hongo. Posteriormente genera una clavija de penetración delgada (Bailey & Jeger, 1978; Perfect et al.,

1999; Politis & Wheeler, 1973; Wharton & Schilder, 2008). A continuación, comienza a desarrollar una hifa primaria que crece intracelularmente y que se propaga y extiende durante la etapa biotrófica. Durante este periodo no se observan síntomas de la enfermedad y el hongo se nutre a partir de las células vivas. Posteriormente, a partir de las hifas primarias genera unas hifas secundarias delgadas que se extienden a través del tejido infectado y que ocasionan la muerte celular gracias a la producción de una gran cantidad de enzimas y toxinas. Esta etapa se denomina etapa necrotrófica (J. Bailey et al., 1992; Luttrell, 1974; Parbery, 1996).

Existen diferentes estrategias de infección y colonización relacionadas con la etapa hemibiotrófica intracelular en *Colletotrichum*: A) Modelo de *C. destructivum,* B) Modelo de *C. orbiculare* y C) Modelo de *C. graminicola* (Crouch et al., 2014) **(Figura 3)**.

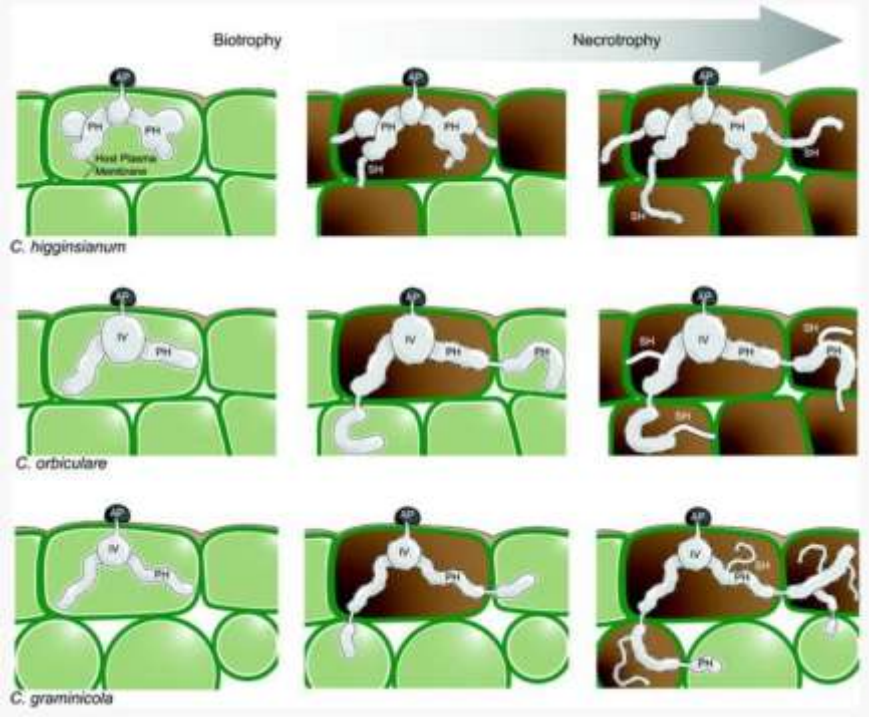


Figura 3. Estrategias de infección y colonización relacionadas con la etapa hemibiotrófica intracelular en *Colletotrichum*. Arriba, modelo de *C. destructivum.* Centro, modelo de *C. orbiculare*. Abajo, modelo de *C. graminicola.* AP = apresorio, IV = vesícula de infección, PH = hifa primaria, y SH = hifa secundaria. Esta figura ha sido extraída del estudio de Crouch et al., 2014.

A)Modelo de *C. destructivum*. La fase biotrófica se encuentra limitada a las primeras células epidérmicas que el hongo infecta. Posteriormente se cambia a la fase necrotrófica produciendo la muerte celular según va avanzando la infección. Este tipo de estrategia es típico de la especie *C. higginsianum* y de otras especies encontradas en el complejo destructivum (Latunde-Dada & Lucas, 2007; O'Connell et al., 2004; O'Connell et al., 2012).

B)Modelo de *C. orbiculare*. La etapa biotrófica es mantenida a medida que el hongo va colonizando las células vegetales secuencialmente. Durante esta fase no se producen los síntomas de la enfermedad. Posteriormente, cambia a la fase necrotrófica donde el hongo genera las hifas secundarias que ocasionan la muerte de las células según se va produciendo su avance. En este etapa, los síntomas de la enfermedad son visibles. Este tipo de estrategia se

encuentra en las especies del complejo orbiculare (R. O' Connell et al., 2000; Perfect et al., 1999; Perfect & Green, 2001).

C)Modelo de *C. graminicola*. Esta estrategia es parecida a la encontrada en las especies del complejo orbiculare, pero con diferencias durante la fase necrotrófica ya que desde las hifas primarias se generan ramas de hifas secundarias necrotróficas y se producen los síntomas de la enfermedad. Al mismo tiempo, las hifas primarias pueden seguir colonizando nuevas células en los bordes de la colonia, de tal forma, que ambas fases conviven durante el desarrollo del hongo. Esta estrategia es típica de las especies del complejo graminicola (Mims & Vaillancourt, 2002; Wharton et al., 2001; Wharton & Julian, 1996).

Ciclo de vida general en el género *Colletotrichum*

El ciclo de vida de las especies del género *Colletotrichum,* ocurre mediante dos fases: asexual o estado anamórfico y sexual o estado teleomórfico **(Figura 4)**. Ambas son llevadas a cabo bien en el huésped o en los restos y desechos de plantas (De Silva et al., 2017; Sutton, 1992). La fase asexual es la forma más habitual y se encuentra relacionado con el desarrollo de la infección y la enfermedad. Por otro lado, la fase sexual es poco frecuente y difícil de observar en la naturaleza (Chona & Bajaj, 1953; Mishra, 1957; Politis, 1975; Vaillancourt & Hanau, 1991), aunque existen algunas excepciones en especies de los complejos gloeosporioides, boninense, destructivum y graminicola. Estos son capaces de producir el peritecio, que es la estructura de reproducción sexual (Ishikawa et al., 2010; Manandhar et al., 1986; Rodriguez et al., 2005; Vaillancourt et al., 2000). Durante la reproducción sexual, se han encontrado estirpes que pueden ser homotálicas o heterotálicas (Chilton & Wheeler, 1949; Wharton & Diéguez-Uribeondo, 2004; Wheeler, 1954). Las especies de este género utilizan un sistema de reproducción sexual que no es representativo de muchos de los miembros de los ascomicetos (sistema MAT1-1/2), ya que utilizan un único gen, *MAT1-2-1* (Liang et al., 2021; Menat et al., 2016; Rodríguez-Guerra et al., 2005; Talhinhas & Baroncelli, 2021; Vaillancourt et al., 2000; Wilson et al., 2021). La variabilidad genética entre las especies puede aumentar gracias a la reproducción sexual. El ciclo continúa cuando los peritecios forman ascosporas que pueden ser dispersadas por el aire (Pinto et al., 2012; Rodriguez et al., 2005; Vaillancourt et al., 2000) o bien, sirven como estructuras de resistencia que permiten al hongo sobrevivir durante periodos desfavorables o que no se encuentra con su huésped. Cuando las condiciones son propicias, las ascosporas pueden germinar en su huésped y ocasionan la infección (G.-H. Kim et al., 2002; Ureña-Padilla et al., 2002). Se forman los acérvulos donde se encuentran los conidióforos. Estos producen una gran cantidad de conidios (esporas resultantes de la reproducción asexual) (V. Kumar et al., 2001). Gracias al viento y las precipitaciones, las esporas pueden ser dispersadas a otros lugares de la planta como las flores, frutos y hojas que no están infectadas. Así el patógeno asegura que su ciclo continue, generando más esporas (Bergstrom & Nicholson, 1999).

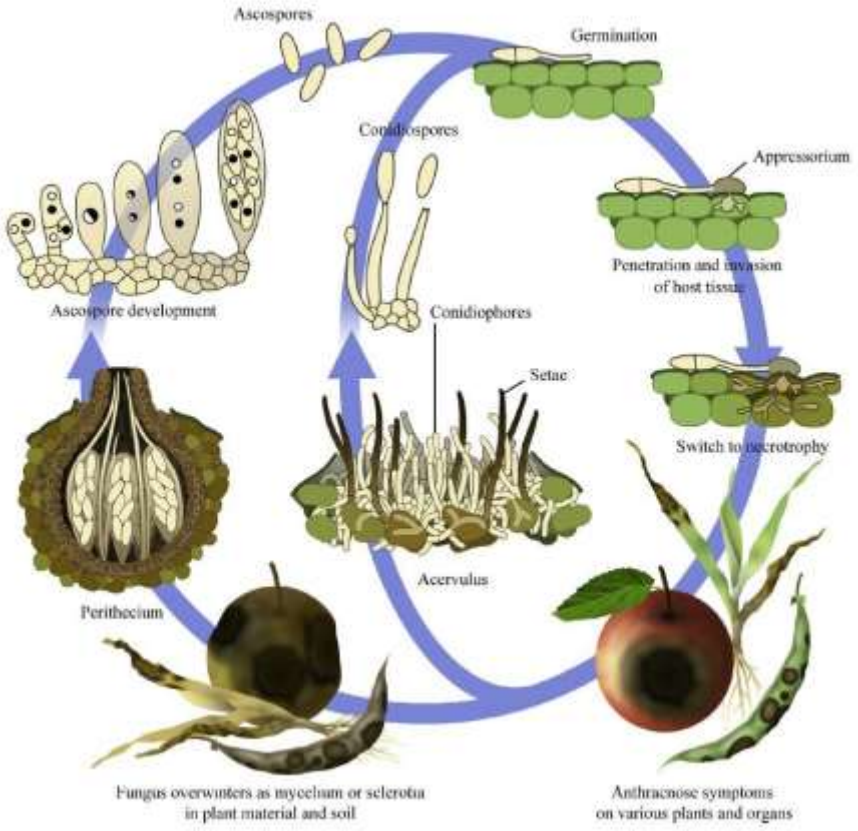


Figura 4. Ciclo de vida de especies del género *Colletotrichum.* El ciclo comienza cuando la espora germina y genera un apresorio que le permite penetrar e invadir el tejido del huésped durante la etapa biotrófica. Posteriormente cambia a la etapa necrotrófica y los síntomas de la antracnosis son visibles. Se generan los acérvulos, que es una estructura donde se encuentran los conidióforos. Estos producen las conidiósporas que pueden volver a comenzar el ciclo (reproducción asexual). En cuanto a la reproducción sexual, el hongo desarrolla los peritecios que generan ascosporas con las que puede iniciar nuevamente la infección. Esta figura ha sido extraída del estudio de De Silva et al., 2017.

La antracnosis del maíz es ocasionada por el hongo *Colletotrichum graminicola*

La especie *Colletotrichum graminicola* (Ces.) G.W Wils (Wilson, 1914) se encuentra agrupada dentro del complejo graminicola. Este complejo está compuesto por otras 15 especies más que se caracterizan por ser patógenas de gramíneas y producir esporas con forma fusiforme (Talhinhas & Baroncelli, 2021). La especie *C. graminicola* divergió hace 12-16 millones de años , durante la época del Mioceno (periodo Neógeno, era Cenozoica) (Bhunjun et al., 2021). Este hongo ocasiona la enfermedad que es conocida como antracnosis del maíz (*Zea mays*) cuyos principales síntomas son la pudrición del tallo y el tizón foliar (Bergstrom & Nicholson, 1999; Jamil & Nicholson, 1991).

C. graminicola puede causar grandes pérdidas económicas a nivel mundial en los cultivos de maíz (Callaway et al., 1992; Cota et al., 2012; Perkins & Hooker, 1979; Smith, 1976). En la última década se han publicado dos informes determinando las pérdidas que pueden llegar a ocasionar la antracnosis del maíz en Estados Unidos y Ontario (Canadá), siendo más severa la pudrición del tallo que el tizón foliar. Se han cuantificado aproximadamente pérdidas por valor de unas 38.000 toneladas de maíz (938,7 millones de fanegas) entre los años 2012 y 2019 (D. S. Mueller et al., 2016, 2020). Además, análisis experimentales sobre los daños que puede ocasionar la pudrición del tallo del maíz demostraron que producían una disminución del peso del grano de

maíz (entre un 20 y 25%), tamaño de la inflorescencia femenina (entre un 6 y 8%) y su peso (entre un 20 y 23%). Del mismo modo, se vio una reducción en el contenido de clorofilas y alteraciones en el fotosistema II (L. J. M. Campos et al., 2021). Por otro lado, como consecuencia del cambio climático y las transformaciones que se están dando en las prácticas agrícolas, el impacto de la antracnosis del maíz podría aumentar en el futuro (Frey et al., 2011; Munkvold, 2002; Sanz-Martín, et al., 2016a).

El maíz es un cultivo importante para la seguridad alimentaria global, sin embargo, la producción mundial puede verse afectada por los fitopatógenos (Miedaner & Juroszek, 2021; D. S. Mueller et al., 2020). Este cultivo es el segundo de mayor importancia en la Unión Europea tras el trigo. Se estima que, en 2019, se obtuvieron 8,9 millones de hectáreas dedicadas al cultivo de grano de maíz y 6,4 millones de hectáreas para maíz ensilado (verde) (EUROSTAT, 2020b, 2020a; Miedaner & Juroszek, 2021). Con respecto a España, durante el año 2020, se dedicaron 325.000 hectáreas de cultivo de maíz siendo Castilla y León el área productora de mayor importancia con un 35,2% de la productividad nacional (MAPA, 2021). Como métodos de contra el hongo *C. graminicola,* en maíz, se aconseja el uso de variedades de maíz resistentes o híbridas, así como la rotación de cultivos (Cota et al., 2012; Crouch & Beirn, 2009). Se pueden utilizar plantas que no pertenezcan a la familia de las gramíneas, como la soja (leguminosa) (Jirak-Peterson & Esker, 2011). También se recomienda realizar un manejo adecuado del campo tras el cosechado como, la recogida de los residuos o el labrado del campo, que ayudan a la descomposición de los restos de maíz (Crouch & Beirn, 2009; Jirak-Peterson & Esker, 2011). Por último, se puede reducir los daños producidos por la antracnosis del maíz si se limitan otros estreses que puedan afectar a la planta como, estreses abióticos y bióticos (nemátodos de las raíces, insectos perforadores, entre otros) (Perrine-Walker & Anderson, 2019).

Características genómicas de *Colletotrichum graminicola*

La especie *C. graminicola,* fue la primera especie del género *Colletotrichum* que fue secuenciado su genoma (O'Connell et al., 2012). El genoma de *C. graminicola* estirpe M1.001 (número de acceso NCBI: ACOD00000000.1) tiene un tamaño estimado de 57,4 megabases. Gracias al genoma y un mapa óptico, se concluyó que el genoma estaba dispuesto en 13 cromosomas, de los cuales 3 eran minicromosomas con un tamaño menor a 1 megabase. Se estima que el genoma se compone de regiones repetitivas en un 12,2% que tienen bajo contenido en GC. Sin embargo, si se tienen en cuenta las regiones que no fueron posibles de ensamblar, el porcentaje aumenta hasta un 22,3%. Estas regiones están compuestas por el ADN ribosómico, telómeros, centrómeros y transposones. Además, se observó que los minicromosomas contienen un alto porcentaje de ADN repetitivo.. En el estudio de Taga y colaboradores (Taga et al. 2015) se realizó un análisis del cariotipo de varias especies de *Colletotrichum*. Los núcleos de *C. graminicola* fueron teñidos con el colorante fluorescente DAPI durante la prometafase **(Figura 5)**.

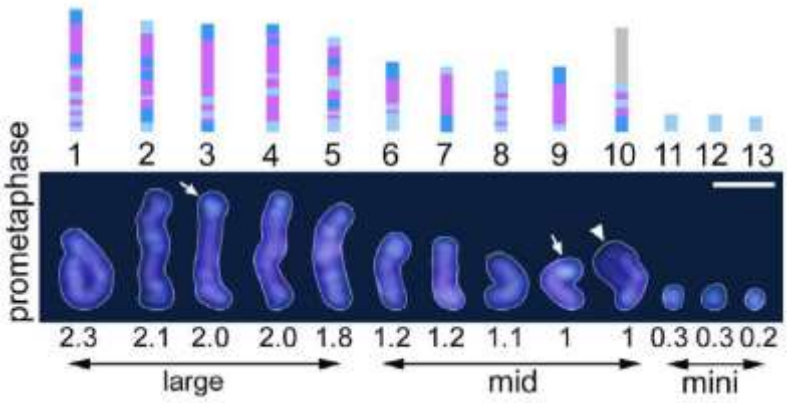


Figura 5. Cariotipo de la estirpe *C. graminicola* M1.001 durante la prometafase. Se observan 13 cromosomas en los que se describe su tamaño, número y posición: 5 cromosomas grandes (*large*), 5 cromosomas medianos (*mid*) y 3 cromosomas pequeños o minicromosomas (*mini*). Los números debajo del diagrama de los cromosomas representan la longitud relativa del cromosoma. La barra de escala es de 2 µm. Los cromosomas fueron teñidos con el colorante fluorescente DAPI. Se pueden observan bandas de diferentes intensidades: de azul oscuro se representan aquellas de mayor intensidad, y con azul claro las de menor. Las flechas en blanco muestran nudos cromosómicos, mientras que la flecha sin punta, indica la región organizadora nucleolar. Esta figura ha sido extraída del estudio de Taga et al., 2015.

Colletotrichum graminicola tiene una distribución mundial

La especie *C. graminicola* se encuentra distribuida alrededor del planeta (Sanz-Martín et al., 2016a). Gracias a la base de datos del *Invasive Species Compendium* (CABI, 2021), se pueden encontrar los países donde se ha descrito la presencia de *C. graminicola* **(Figura 6).**

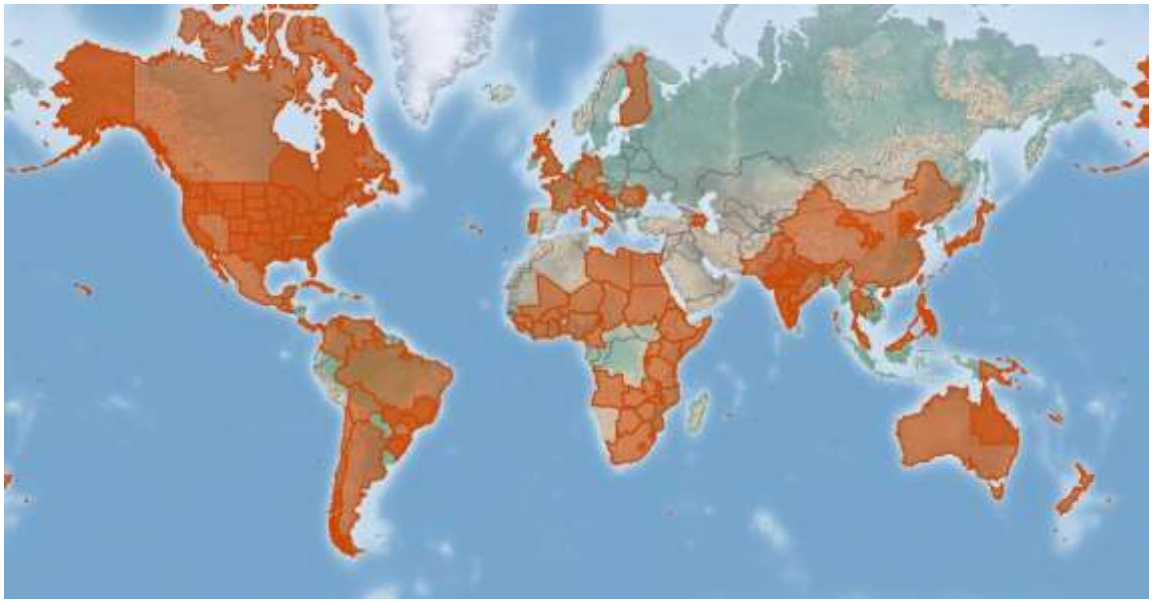


Figura 6. Distribución de *C. graminicola* en el mundo. Este mapa ha sido extraído desde la base de datos de *Invasive Species Compendium* (CABI, 2021).

En Europa, aparece en 14 países en la actualidad: Austria, Bosnia y Herzegovina, Croacia, Finlandia, Francia, Alemania, Moldavia, Portugal, Rumanía, Serbia, Montenegro, Eslovenia, Suiza y Reino Unido (Böning & Wallner, 1936; CABI & EPPO, 2005; Chowdhury, 1936; Cuevas-Fernández et al., 2019; Messiaen, 1954; Palaversic et al., 2009; Quebral, 1958; Sanz-Martín et

al., 2016b; Sukno et al., 2014; Winter & Menzi, 1991). También fue encontrado la ex Checoslovaquia (Sychrova et al., 1974). Uno de los países donde todavía no se ha descrito es España, aunque según una comunicación personal de Pionner Hi-Bred (España), se ha encontrado en la provincia de Lérida (Sanz-Martín, 2016) y esporádicamente se observa en Galicia (comunicación personal Betran, J., Bayer). Recientemente se ha sido descrito en otras partes del mundo como en China (Duan et al., 2019).

Expansión de *C. graminicola* en Europa, Bosnia y Herzegovina

Debido a que *C. graminicola* supone una amenaza para los cultivos de maíz (Gorman et al., 2020; D. S. Mueller et al., 2020), el estudio de la propagación de *C. graminicola* es de vital importancia. Durante el año 2019 se realizó la primera descripción de la especie *C. graminicola* en Bosnia y Herzegovina (Cuevas-Fernández et al., 2019), cuya publicación se encuentra en el Capítulo IV de esta tesis. Bosnia y Herzegovina es un país situado en el sureste de Europa en la Península de los Balcanes **(Figura 7A)**. Sus países vecinos son Croacia, Serbia y Montenegro. La economía de este país está basada fundamentalmente en el sector servicios, la industria y la agricultura. Esta última representó un 6,8% del producto interior bruto del país en el año 2017 (MAEUEC, 2021). Con respecto al cultivo del maíz, es el decimosexto productor de Europa con un valor aproximado de 1,23 millones de toneladas y 192.000 hectáreas de cultivo en el año 2019 (FAOSTAT, 2021) **(Figura 7B)**.

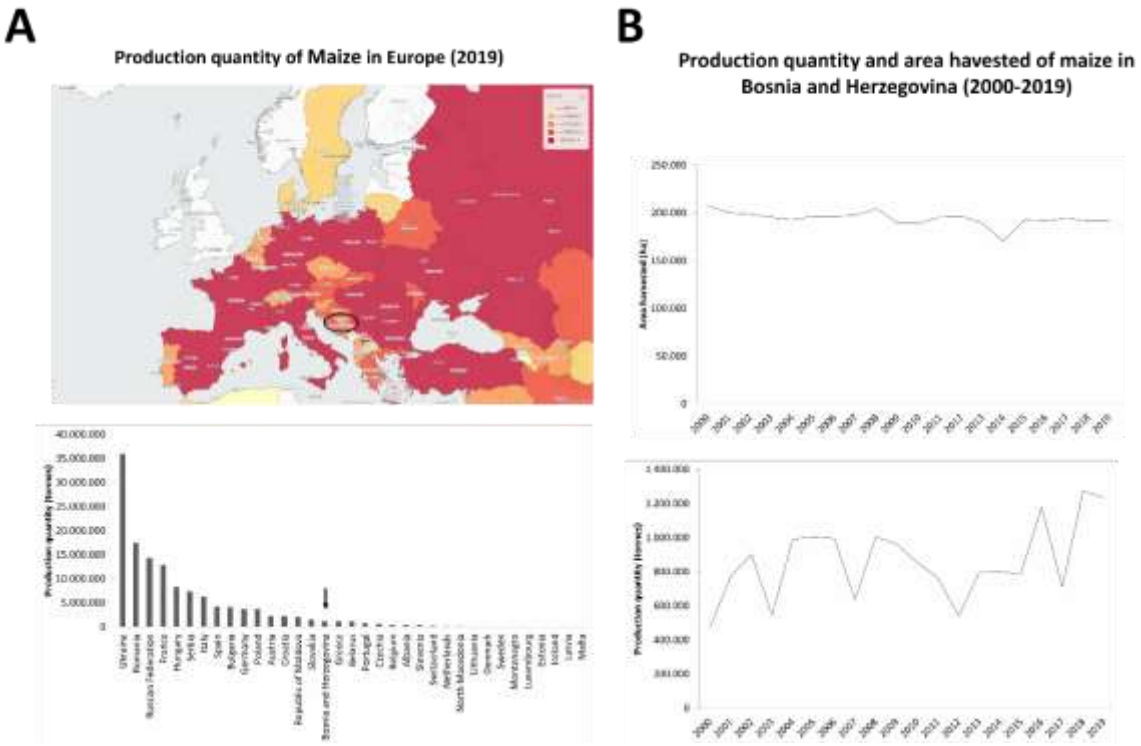


Figura 7. Producción de maíz en Bosnia y Herzegovina. A) Mapa de producción de maíz (arriba) y gráfico de comparación de la producción con el resto de los países europeos (abajo) durante el año 2019. En el mapa se puede ver la situación de Bosnia y Herzegovina rodeada con un círculo. Este país es el decimosexto productor de Europa que está señalado con una flecha en el gráfico. **B)** Datos de superficie cultiva de maíz (arriba) y producción (abajo) durante el periodo 2000-2019 en Bosnia y Herzegovina. El mapa, los datos de producción y área cultivada han sido extraídos de la base de datos FAOSTAT (FAOSTAT, 2021).

Los hongos pueden trasladarse a través del mundo mediante múltiples formas. Un hongo podría llegar a un nuevo país a través del transporte de plantas o semillas (Sikes et al., 2018; Warren &

Nicholson, 1975). Este es capaz de vivir de manera endofítica en la planta sin producir síntomas. Posteriormente puede desplazarse al huésped del cual es patógeno. La producción de diferentes tipos de esporas que son transportadas a largas distancias por el aire o por insectos, la propagación vegetativa, entre otros, pueden ser factores que ayuden a su dispersión. Además, las esporas también podrían diseminarse a través de la ropa, vehículos, paquetes y pájaros (Willis, 2018). Estos factores podrían favorecer la dispersión de *C. graminicola* lo que supondría un peligro para países próximos a Bosnia y Herzegovina como Hungría, que fue el quinto productor de maíz de Europa durante el año 2019 (FAOSTAT, 2021)y donde todavía no ha sido reportada la enfermedad.

Ciclo de vida de *Colletotrichum graminicola* en el maíz

El ciclo de vida de *C. graminicola* se resume en las siguientes fases: hibernación del inóculo, propagación, proceso infectivo de hojas, tallos y raíces; y, por último, crecimiento saprofítico (Bergstrom & Nicholson, 1999; Crouch & Beirn, 2009; Perfect et al., 1999). Se abordará también el estado sexual.

1)Hibernación del inóculo. *C. graminicola* es capaz de hibernar durante épocas desfavorables como el invierno en los restos vegetales que se encuentran en el suelo en diferentes formas como: micelio, acérvulos, hipopodios que se encuentran melanizados y microesclerocios (Bergstrom & Nicholson, 1999; Casela & Frederiksen, 1993; Crouch & Beirn, 2009; Serenella A. Sukno et al., 2008). Estas formas sirven a *C. graminicola* como fuente de inóculo primario y suelen son más virulentas que otras fuentes (Casela & Frederiksen, 1993; Crouch & Beirn, 2009; P E Lipps, 1983, 1985, 1988; Naylor & Leonard, 1977). Existen diferentes factores que influyen en la supervivencia de *C. graminicola* en el suelo como, las condiciones medioambientales, la temperatura o la relación con otros microorganismos (Crouch & Beirn, 2009). Se estima que *C. graminicola* puede resistir en los restos vegetales durante 20 meses (Naylor and Leonard, 1977; Crouch & Beirn, 2009).

2)Propagación. Las esporas con forma fusiforme (asexuales), son capaces de diseminarse mediante el viento y también a través de las gotas de lluvia (Bergstrom & Nicholson, 1999; Crouch & Beirn, 2009). A su vez, las esporas fusiformes fusiformes producidas en masa a través de los acérvulos que se desarrollan sobre tejidos necrosados de la planta pueden actuar como una fuente de inóculo secundario (Crouch & Beirn, 2009).

3)Proceso infectivo. *C. graminicola* es una especie que se ha utilizado como modelo para estudiar los hongos fitopatógenos con estilo de vida hemibiotrófico (Bergstrom & Nicholson, 1999; Crouch & Beirn, 2009; Perfect et al., 1999; Vargas et al., 2016). *C. graminicola* se asocia con la estrategia de hemibiotrofía intracelular descrito anteriormente **(Figura 3)**.

3A) Infecciones de hojas o tizón foliar del maíz **(Figuras 8 y 9)**. El proceso infectivo comienza cuando la espora llega a la hoja del maíz. La espora está recubierta por una matriz extracelular que le permite adherirse a la superficie de la hoja. La matriz se encuentra formada por compuestos como manosa y otras glicoproteínas con un alto grado de glicosilación (Sugui et al., 1998; Crouch and Beirn, 2009). Después, la espora germina y se genera el tubo germinativo (Crouch & Beirn, 2009; Mercure et al., 1994; Sugui et al., 1998). La germinación ocurre a las 12 horas después de la infección (Vargas et al., 2012). Posteriormente se forma el apresorio que está melanizado sobre la superficie de la hoja a las 24 horas después de la infección. A continuación, el hongo desarrolla una clavija de penetración delgada con la que atraviesa las

células epidérmicas (Politis and Wheller, 1972; Mims & Vaillancourt, 2002; Vargas et al., 2012). Durante este periodo el hongo genera una gran fuerza sobre la pared celular, además de liberar de forma muy regulada enzimas degradadoras de la pared celular, que facilitan su deterioro (Kleemann et al., 2008; O' Connell et al., 2012; Vargas et al., 2016). La penetración del hongo también podría darse a través de heridas que se han podido producir de manera natural en la planta (Crouch & Beirn, 2009; Venard & Vaillancourt, 2007). Dentro de las células epidérmicas, se forman las hifas primarias durante un periodo que va desde las 24 a 36 horas después de la infección. Las hifas primarias tienen un aspecto irregular y están engrosadas. En este momento comienza la etapa biotrófica del hongo en la que no se observan síntomas de la enfermedad y las células se mantienen vivas. Después, las hifas primarias siguen creciendo y progresando hacia otras células que no están infectadas durante las 36 a 60 horas después de la infección. (Bergstrom & Nicholson, 1999; Micali et al., 2011; Panstruga, 2003; Perfect & Green, 2001; Vargas et al., 2012). Tras esta etapa, las hifas secundarias son formadas a partir de las células primarias aproximadamente las 60-70 horas después de la infección. La duración de esta etapa depende del huésped y de las condiciones ambientales. Las hifas secundarias tienen un aspecto regular y son pequeñas y delgadas. Estas son capaces de crecer intra e intercelularmente en el tejido de la planta. Finalmente, se desarrollan las lesiones necróticas que son visibles gracias al crecimiento generalizado de las hifas secundarias, que se corresponden con la etapa necrotrófica de la antracnosis del maíz (Bergstrom & Nicholson, 1999; Mims & Vaillancourt, 2002; O' Connell et al., 1985; Vargas et al., 2012; Wharton et al., 2001).

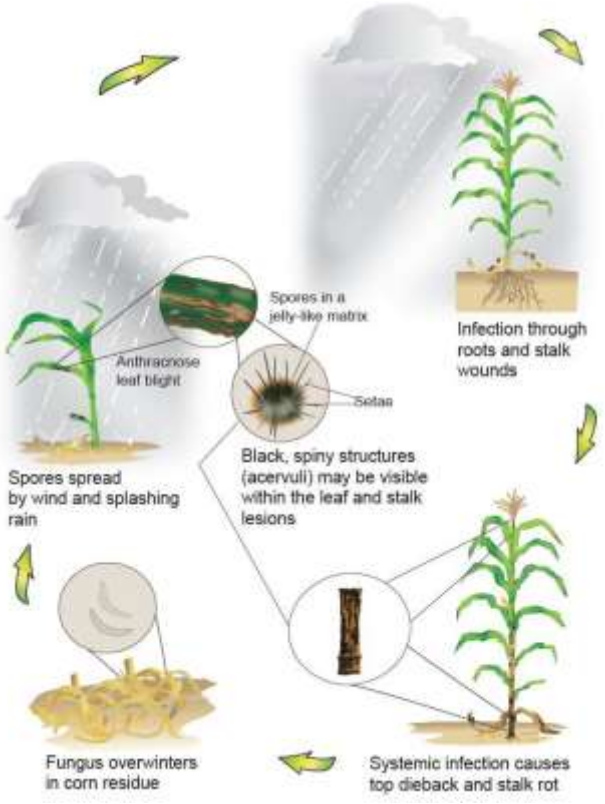


Figura 8. Infección de hojas y tallos de maíz por el hongo *C. graminicola*. Los desechos de plantas en el suelo sirven como fuente de inóculo primario de *C. graminicola* que puede causar la infección de plántulas jóvenes de maíz. Se ocasiona el tizón foliar (*leaf bligth*). Las esporas producidas en los acérvulos pueden servir de inóculo secundario y trasladarse a otras regiones de la planta como el tallo a través del viento y la lluvia. La infección en el tallo produce síntomas de podredumbre (*stalk rot*). Las infecciones sistémicas pueden ocasionar la muerte regresiva (*top dieback*). Por otro lado, el maíz también puede ser infectado a través de las raíces o heridas en los tallos. Esta figura ha sido extraída de la base de datos de *Crop Protection Network* (CNP, 2021).

Figura 9. Infección de hojas de maíz. Síntomas del tizón foliar. Se producen lesiones necróticas y clorosis sobre las hojas. Fotografías realizadas por A. Roberson y extraídas de la base de datos de *Crop Protection Network* (CNP, 2021)

3B) Infección a través de los tallos o pudrición del tallo **(Figuras 8 y 10)**. El proceso infectivo en los tallos es parecido a la infección que se produce en las hojas, por lo que podría tratarse de una prolongación de esta (Bergstrom & Nicholson, 1999; Venard & Vaillancourt, 2007). La infección del tallo puede darse a través de superficies intactas, por heridas o por insectos (Venard & Vaillancourt, 2007). El proceso empieza cuando las esporas que han sido producidas por infecciones en las hojas, son arrastradas por el agua a través de la vaina de la hoja. Estas llegan a la corteza del tallo donde se inicia la infección (Bergstrom & Nicholson, 1999). A las 24 horas después de la infección se producen los apresorios en la superficie de las células epidérmicas y, a las 48 horas después de la infección, se observan las hifas primarias. A los dos días después de la infección, en las células epidérmicas se observa una red de hifas primarias que se propagan entre las células por conexiones muy estrechas. Posteriormente se desarrolla un estroma donde se generan los acérvulos y los conidios, uno o dos días después de la infección. A continuación, se produce la propagación del hongo hacia las fibras de la corteza y el parénquima tras el paso de dos días más. Finalmente *C. graminicola* provoca la pudrición de las células del tallo (con la excepción de las células epidérmicas y las fibras de la corteza) (Venard & Vaillancourt, 2007). *C. graminicola* también es un patógeno que es capaz de crecer en el sistema vascular de la planta, propagándose de manera sistémica. Es lo que se conoce como "muerte regresiva del tallo", cuyos síntomas principales son la muerte de los internodos y las hojas superiores del tallo (que ocurre durante las etapas tempranas del desarrollo del grano en las mazorcas) (Smith & White, 1988; White, 1999; White et al., 1979). Sin embargo, estos síntomas no son observables hasta que la planta se vuelve senescente (Bergstrom & Nicholson, 1999; Keller et al., 1986). El crecimiento por el sistema vascular también puede darse a través de las raíces (Sukno et al., 2008).

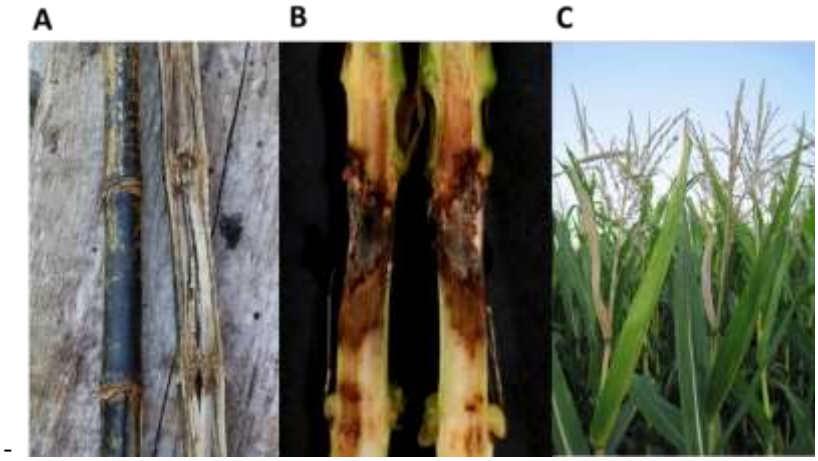


Figura 10. Infección de tallos de maíz. A, B) Síntomas de la podredumbre del tallo. **C)** Síntomas de la muerte regresiva del tallo. Las fotografías A (A. Sisson) y C (A. Anderson) han sido extraídas de la base de datos de *Crop Protection Network* (CNP, 2021). La fotografía B, fue tomada en el laboratorio durante uno de los experimentos de infección de tallos tras 14 días después de la infección.

3C) Infección a través de las raíces **(Figura 11)**. *C. graminicola* es capaz de crecer mediante un modelo distinto a los vistos anteriormente (propagación en la raíz célula a célula). El hongo coloniza la raíz siguiendo un patrón en forma de mosaico en las células epidérmicas y corticales. Se forman hifas primarias o corredoras, hipopodios y microesclerocios. Los síntomas de la enfermedad son muy tardíos y no aparecen hasta 42 días después de la infección. Otra particularidad es que *C. graminicola* puede crecer desde las raíces y acceder al sistema vascular, mediante el cual se expande hasta llegar a las regiones aéreas de la planta. Se produce una infección sistémica que no produce síntomas (Sukno et al., 2008).

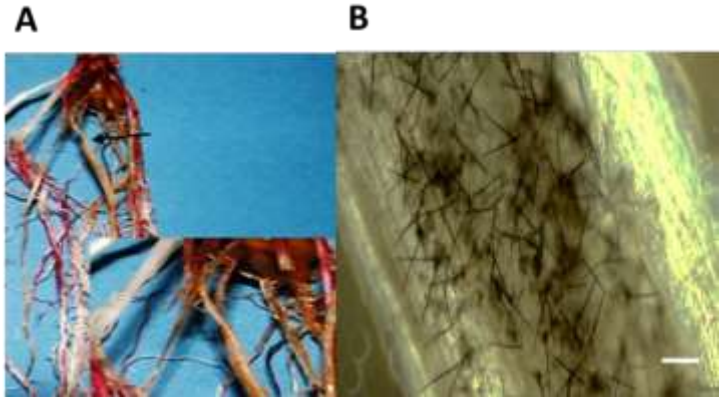


Figura 11. Infección raíces de maíz. A) Se produce un cambio de coloración (marrón) en las raíces (flecha negra) que están infectadas con *C. graminicola* tras 28 días después de la infección. **B)** Raíz donde se han desarrollado los acérvulos. Esta figura ha sido extraída del estudio de Sukno et al., 2008.

4)Crecimiento saprofítico. El hongo es capaz de desarrollarse de manera saprófita en los restos vegetales de maíz infectados que se encuentran sobre el suelo. La hibernación del hongo se alarga hasta la primavera cuando se da la fase de esporulación. En los tallos infectados se generan las esporas que están rodeadas por un mucílago extracelular que les ayuda a refugiarse frente a condiciones ambientales perjudiciales como la desecación. Las esporas permanecen en este estado hasta que vuelven a tener disponibles plántulas de maíz a las que poder infectar de nuevo (Bergstrom & Nicholson, 1999).

5)Estado sexual. El estado teleomorfo de *C. graminicola* es *Glomerella graminicola* D. J. Politis (Politis, 1975). Este fue descrito en condiciones de laboratorio, sin embargo, no ha sido posible encontrarlo en condiciones de campo aún (Crouch & Beirn, 2009; Vaillancourt & Hanau, 1992).

Colletotrichum graminicola genera dos tipos de esporas diferentes

El hongo *C. graminicola* es capaz de producir dos tipos de esporas asexuales que se distinguen según su forma: esporas fusiformes y ovaladas (Nordzieke et al., 2019; Panaccione et al., 1989). En general, las esporas fusiformes son las que se encuentran en mayor proporción durante el proceso infectivo. Estas se generan en los tejidos necrosados de las plantas de maíz (Bergstrom & Nicholson, 1999; Crouch & Beirn, 2009). Por otro lado, las esporas ovaladas son más pequeñas y se producen durante la infección del sistema vascular del maíz. Quizás, estas esporas pueden ocasionar la distribución de la enfermedad en la planta (Nodrzieke et al., 2019). Aunque ambos tipos de esporas han sido estudiados desde hace décadas, se sabe poco acerca de cuál es el papel que juegan durante el proceso infectivo (Panaccione et al., 1989; Sukno et al., 2008; Nodrzieke et al., 2019). En el estudio de Nodzieke y colaboradores (Nodrzieke et al. 2019) se realizaron algunos nuevos descubrimientos del ciclo de vida y la patogenicidad de las esporas fusiformes y ovaladas **(Figura 12)**. En cuanto a la germinación, las esporas fusiformes liberan al medio la micosporina-glutamina, que inhibe el crecimiento del resto de las esporas. Las esporas ovaladas, no son capaces de producir esto compuesto. La micosporina-glutamina puede ser producida durante periodos de falta de nutrientes, que impide la germinación del resto de las esporas. Por el contrario, las esporas ovaladas pueden germinar más rápido en condiciones de bajos nutrientes (Chaky et al., 2001; Leite & Nicholson, 1992; Nordzieke et al., 2019). La generación de tubos de anastomosis entre las esporas en agua es un proceso común en los hongos ascomicetos. Las esporas asexuales son capaces de fusionarse a través de la generación de unas hifas específicas que permite la interconexión de ambas esporas. Estas a su vez, pueden fusionarse a las líneas germinales derivadas formando una red (Nordzieke et al., 2019; Read et al., 2012). Las esporas ovaladas y las líneas germinales derivadas tienen una tasa muy alta de formación de tubos de anastomosis, lo que les permite fusionarse entre ellas. Por otro lado, este proceso no se contempla en las esporas fusiformes. La fusión de las esporas ovaladas es muy importante durante la infección del maíz, ya que en aquellas en las que se prevenía este proceso, se veía reducida la formación de los hipopodios por parte de las hifas (Nodrzieke et al., 2019). Esta es la estructura de penetración que se forma en presencia de raíces (Sukno et al, 2008). Sin embargo, en las esporas fusiformes no es importante la formación de tubos de anastomosis ya que son capaces de generar los apresorios que les permite penetrar directamente las hojas (Nodzieke et al., 2019).

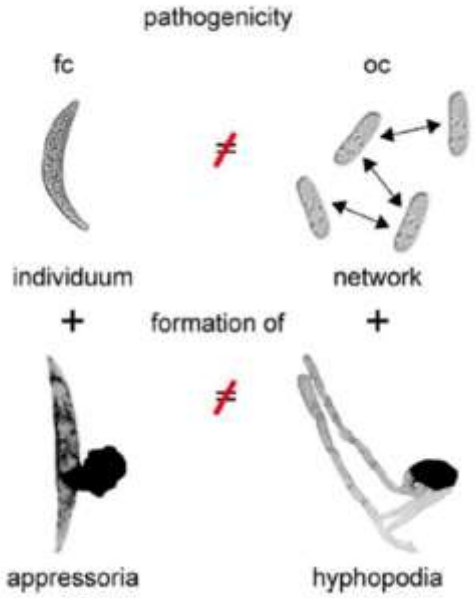


Figura 12. Diferentes esporas asexuales producidas por *C. graminicola* y su papel durante la patogénesis. Se observan las esporas con forma fusiforme (arriba izquierda) y ovalada (arriba derecha). La patogenicidad de las esporas ovaladas depende de la generación de los tubos de anastomosis y fusión, mientras que en las esporas fusiformes no. Las esporas fusiformes producen apresorios (abajo izquierda) y las esporas ovaladas producen hipopodios (abajo derecha). Esta figura ha sido extraída del estudio de Nodrzieke et al., 2019.

El sistema inmunitario de las plantas

Las plantas se encuentran sometidas a la acción de numerosos microorganismos patogénicos. En consecuencia, han desarrollado un complejo sistema inmune que es capaz de detectar a los patógenos y como resultado, se desencadena la respuesta de defensa. Para ello, las plantas utilizan los receptores de reconocimiento de patrones (PRRs) que se encuentran en la membrana plasmática que reconocen patrones moleculares asociados a patógenos (PAMPs) o patrones moleculares asociados a microorganismos (MAMPs) (Y. Peng et al., 2018; X. Yu et al., 2017) como (Y. Lu & Tsuda, 2021) la flagelina (Felix et al., 1999), el factor de elongación Tu (Kunze et al., 2004) o la quitina de los hongos (Shibuya & Minami, 2001)**(Figura 14)**. También pueden detectar patrones moleculares asociados a daño (DAMPs) que derivan de la propia planta y le sirven como una señal de amenaza frente a los daños producidos por el patógeno. Los DAMPs pueden ser proteínas citosólicas, péptidos, nucleótidos y aminoácidos (S. Hou et al., 2019). Cuando los PAMPs/MAMPs se unen a los PRRs se desencadena la inmunidad provocada por P/MAMPs (PTI) (J. D. G. Jones & Dangl, 2006; Y. Lu & Tsuda, 2021). Del mismo modo, también se puede ocasionar la inmunidad asociada a DAMPs que está solapada con la respuesta de señalización PTI (Boller & Felix, 2009; S. Hou et al., 2019; Lotze et al., 2007) **(Figura 13A)**. De manera general se produce la activación de proteínas quinasas activadas por mitógeno (MAPKs), la explosión oxidativa, cambios en la entrada de flujo de iones, el aumento de la producción de hormonas que participan en la defensa, entre otros (Y. Peng et al., 2018). La respuesta PTI puede ser contrarrestada por el patógeno gracias a la liberación de efectores, lo que se conoce como susceptibilidad desencadenada por efectores **(Figura 13B)**. Sin embargo, la planta puede activar una segunda respuesta de defensa mediante el reconocimiento de los efectores y se conoce como respuesta desencadenada por efectores (ETI) (J. D. G. Jones & Dangl, 2006; Y. Lu & Tsuda, 2021) **(Figura 13C)**. Generalmente, esta respuesta es muy específica y es mucho más fuerte que la respuesta PTI, pudiendo desencadenar la respuesta hipersensible (Y. Peng et al., 2018). Recientemente se ha visto como ambas rutas de defensa se encuentran solapadas y comparten

una serie de componentes (Y. Lu & Tsuda, 2021). La respuesta de defensa PTI es necesaria para que se produzca por completo la respuesta de defensa ETI. A su vez, la respuesta ETI es capaz de ayudar en la consolidación de componentes de la respuesta PTI. La respuesta ETI también participa en la inducción de genes implicados en la respuesta de defensa y señalización de PTI. Se ha especulado que el aumento de calcio durante la respuesta ETI podría ser el causante del efecto que se observa durante la respuesta PTI (Bjornson & Zipfel, 2021; Ngou et al., 2020; M. Yuan, Jiang, et al., 2021) **(Figura 13C)**.

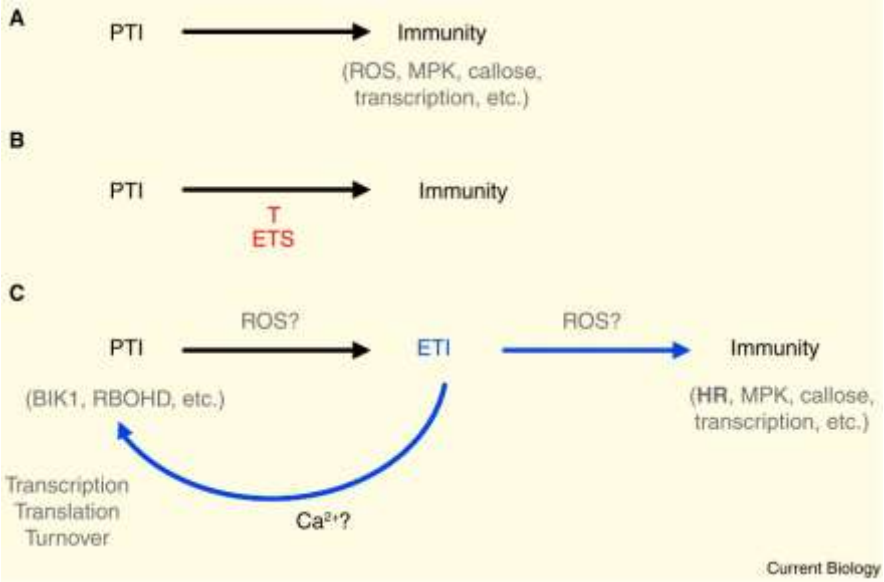


Figura 13. Respuesta inmunitaria de las plantas. A) Respuesta de defensa PTI. Cuando la planta reconoce a un patógeno a través de los PRRs, se produce este tipo de respuesta. Como consecuencia se desencadena la ruta de las MAP kinasas (MPK), la generación de especies reactivas de oxígeno (ROS), deposiciones de callosa, regulación transcripcional, entre otros. **B)** Los patógenos son capaces de liberar efectores que inhiben la respuesta de defensa PTI, por lo que el huésped se da susceptibilidad desencadenada por efectores (ETS). **C)** Respuesta de defensa ETI. Cuando la planta es capaz de reconocer las proteínas efectoras del patógeno, se da este tipo de respuesta. Como resultado, se puede producir la respuesta hipersensible (HR), la activación de las MPK, el aumento de las deposiciones de callosa, la regulación transcripcional, entre otras. Se ha visto que las ROS pueden incrementar estos efectos durante la ETI. A su vez, la respuesta de defensa ETI puede ser inducida a través de un aumento de calcio. Esta figura ha sido extraída del estudio de Bjornson & Zipfel, 2021.

Tipos de receptores durante la respuesta inmunitaria

Los receptores pueden asociarse en dos grupos dependiendo de su localización en la célula. Los PRRs se encuentran en la superficie celular y reconocen ligandos extracelulares. Por otro lado, los receptores intracelulares y de unión a nucleótidos ricos en leucina (NLR) son capaces de detectar a los efectores (Dodds & Rathjen, 2010; Y. Lu & Tsuda, 2021).

Los receptores PRR pueden reunirse en dos grandes grupos. 1) Receptores tipo quinasa (RLKs) cuya estructura está formada por un ectodominio que sirve de unión al ligando, un dominio de paso transmembrana y un dominio quinasa que se ubica en el citoplasma. 2) Receptores tipo proteína (RLPs) que son parecidos a los RLKs, pero a diferencia del dominio quinasa, tienen una pequeña cola (Couto & Zipfel, 2016; Y. Lu & Tsuda, 2021) . Para ambos tipos de receptores, la especificidad se encuentra asociada al ectodominio que contienen en sus estructuras: dominios ricos en leucina (LRR), que se unen a proteínas; dominios motivo de lisina (LysM) que unen

glicanos; dominios de lectina, que se unen a carbohidratos; y dominios de la pared celular asociados a la familia de las quinasas, que unen los oligogalacturonidos (Y. Lu & Tsuda, 2021; Saijo et al., 2018) **(Figura 14).**

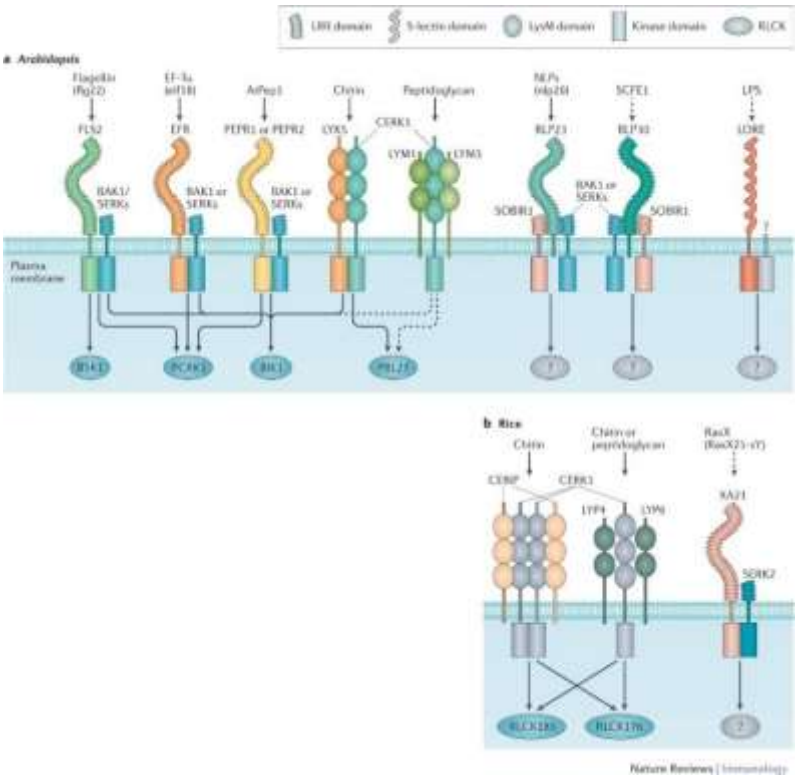


Figura 14. Diferentes receptores PRRs en *Arabidopsis thaliana* (A) y arroz (B). Esta figura muestra un ejemplo de los diferentes receptores PRRs, correceptores, tipo de dominios que conforman su estructura y los ligandos con los cuales pueden interaccionar. Esta figura ha sido extraída del estudio de Couto & Zipfel, 2016.

Además, los RLKs y RLPs se encuentran asociados a su vez a correceptores (Y. Lu & Tsuda, 2021). Se han caracterizado diferentes receptores: el receptor LRR-RLK *Flagelling sensing 2* (FLS2) tiene dos correceptores, *Bri1-associated receptor kinase 1/somatic emrbyogenesis receptor kinase 3* (BAK1/SERK3) y su homólogo BAK1/SERK4 (Chinchilla et al., 2007; Y. Lu & Tsuda, 2021). La flagelina 22 (flg22), que es un epítopo similar a la flagelina de las bacterias, es capaz de provocar la formación de un complejo formado por FLS2-BAK1 a través de los dominios LRR que tiene el receptor FLS2 y su correceptor BAK1 **(Figura 14)**. Gracias a la detección de flg22 por parte de este complejo, se desencadena una señal de transducción aguas abajo a través de la transfosforilación de dicho complejo (Y. Lu & Tsuda, 2021; Schulze et al., 2010; Y. Sun et al., 2013). Otro ejemplo es el receptor LysM-RLK *Chitin elicitor receptor kinase 1* (CERK1), que es un correceptor que detecta la quitina de la pared celular de los hongos y también de los péptidoglicanos de la pared celular bacteriana (Y. Lu & Tsuda, 2021; Miya et al., 2007; Wan et al., 2008; Willmann et al., 2011). En *Arabidopsis thaliana,* después de realizar un tratamiento con quitina se produjo la formación del complejo *Lysin motif receptor kinase 5* (LYK5)-CERK1. También provocó la fosforilación de CERK1. CERK1 reconoce a la quitina a través de sus dominios LysM (Cao, Liang, et al., 2014; T. Liu et al., 2012; Y. Lu & Tsuda, 2021)**(Figura 14)**. En el arroz se ha descubierto el receptor LysM-RLP *Chitin-elicitor binding protein* (CEBiP) es el receptor más importante de quitina que puede formar complejos con CERK1 (Hayafune et al., 2014; Y. Lu & Tsuda, 2021; Shimizu et al., 2010) **(Figura 14)**. En los últimos años se han descubierto otros receptores PPRs implicados en el reconocimiento de hongos que se describen en las revisiones de Albert et al. (2020) y Yu et al. (2021).

Los receptores NLR son capaces de reconocer los efectores que son liberados por los patógenos en el citoplasma de la célula vegetal. Este reconocimiento se realiza de manera directa o indirecta mediante el control de la homeostasis o bien, a través de la alteración de las proteínas unidas con el efector. La estructura de los receptores NLR se compone de un dominio N-terminal que es diferente dependiendo del tipo NLR, un dominio *nucleotidide-binding* (NB) *Apaf1-resistance-CED4* (ARC) que se sitúa en la parte central del receptor y un dominio LRR en la región C-terminal (Y. Lu & Tsuda, 2021; Monteiro & Nishimura, 2018) . Dependiendo del tipo de dominio que se encuentra en la región N-terminal, se pueden clasificar en tres grupos principales: *Coiled-coil* (CC), *Toll interleukin-1 receptor* (TIR) y RPW8 (CC_R). A su vez, los NLR pueden clasificarse en "NLR sensores", que son los que reconocen los efectores del patógeno, o "NLR auxiliares", que ayudan a los NLR sensores en la señalización de la respuesta de defensa (Adachi et al., 2019; Y. Lu & Tsuda, 2021). Un ejemplo se encuentra en las especies bacterianas *Xanthomonas euvesicatoria* y *Pseudomonas psyringae,* son capaces de secretar los efectores XopQ1 y HopQ1, respectivamente. Gracias a la interacción directa del receptor NLR del tipo TIR, Roq1 en plantas de tabaco (*Nicotiana benthamiana*) infectados con estos patógenos, se producía la respuesta hipersensible y la resistencia a la enfermedad. Roq1 es capaz de reconocer a los efectores a partir de sus dominios LRR (Y. Lu & Tsuda, 2021; Schultink et al., 2017).

Tradicionalmente, a los efectores que son reconocidos por el huésped, se les ha denominado proteínas de avirulencia (Avr) (ya que el patógeno se vuelve no virulento a ese genotipo del huésped (Lu & Tsuda, 2021). Estos efectores son detectados por las proteínas de resistencia (R) que activan la respuesta de defensa ETI (Dodds & Rathjen, 2010; Y. Peng et al., 2018). Este proceso es conocido como "resistencia gen a gen", modelo que fue propuesto por Flor (1942). La gran mayoría de las proteínas R son receptores NLR, aunque también existen representantes de las familias de receptores RLK y RLP (Peng et al., 2018). Existen cuatro estrategias diferentes por las cuales las proteínas R son capaces de detectar a las proteínas Avr. 1) Modelo elicitor-receptor: la proteína Avr es detectada de forma directa a través de la proteína R, lo que activa la respuesta de defensa (Catanzariti et al., 2010; Dodds et al., 2006; Y. Jia et al., 2000; Keen, 1990; Petit-Houdenot & Fudal, 2017; Steinbrenner et al., 2015). 2) Modelo del guardián: se da un reconocimiento indirecto entre las proteínas R y Avr. El *guardián* es una proteína a la cual se une el efector. La proteína R es capaz de percibir los cambios que se dan en el *guardián* (Dangl & Jones, 2001; Petit-Houdenot & Fudal, 2017). 3) El modelo del señuelo: el efector se une a una proteína "señuelo", que imita a la proteína diana del efector. Entonces la proteína R puede "atrapar" al efector (Petit-Houdenot & Fudal, 2017; van der Hoorn & Kamoun, 2008). 4) Modelo del señuelo integrado: el efector se une a un "señuelo" que se corresponde con dominios no-canónicos que tiene la estructura de la proteína R. Así, la proteína R actúa como un "señuelo" del efector (Bernoux et al., 2014; Le Roux et al., 2015; Petit-Houdenot & Fudal, 2017; Sarris et al., 2015). Existen numerosos ejemplos de este tipo de interacciones en hongos patógenos de plantas. La especie *Leptosphaeria maculans* es capaz de infectar la planta de colza (*Brassica napus*) y es causante del cancro de la base del tallo de la colza. Se identificó que dos genes *R*, *Rlm1* y *LepR3* eran capaces de detectar a la proteína de avirulencia *AvrLm1* (Larkan et al., 2013; Petit-Houdenot & Fudal, 2017). Por otro lado, el hongo *Magnaporthe oryzae* que es el causante de la piriculariosis del arroz*,* es capaz de reconocer genes de avirulencia gracias a la acción de dos proteínas R. Se han encontrado diferentes interacciones: los genes *R Pik-1* y *Pik-2,* son capaces de detectar el gen de avirulencia *AVR-Pik; Pii-1* y *Pii-2,* reconocen a *AVR-Pii; RGA4* y *RGA5,* detecta a *AVR-Pia,* entre otros (Bernoux et al., 2014; Kanzaki et al., 2012; Okuyama et al., 2011; Petit-Houdenot & Fudal, 2017; Takagi et al., 2013).

Las rutas de señalización de los receptores se interconectan durante la respuesta de defensa

Aunque los receptores PRR y NLR difieren en su localización, tipo de señalización, complejos de receptores asociados y requerimientos genéticos durante la etapa temprana, cada vez más estudios reflejan que sus rutas de señalización se encuentran interconectadas. Ambas presentan componentes comunes pero que se utilizan de manera diferente (Y. Lu & Tsuda, 2021; Mine et al., 2018; Tsuda et al., 2009; Tsuda & Katagiri, 2010). De esta forma, la señalización que depende de los receptores PRR y NLR puede desencadenar numeras respuestas aguas abajo como el influjo de calcio, estallido de ROS, estimulación de las cascadas de MAPKs, activación de la transcripción de genes de defensa y la síntesis de hormonas relacionadas con la defensa (Lu & Tsuda, 2021) **(Figura 15)**.

1)Señalización de calcio. Este catión es considerado como un mensajero secundario en las rutas de señalización de las células de los organismos eucariotas. Participa en el desarrollo de las plantas y ante ciertos estreses, creando flujos de calcio específicos (Y. Lu & Tsuda, 2021; W. Tian et al., 2020). En la planta *A. thaliana,* tras el tratamiento de flg22, se produce su detección a través del PRR FLS2/BIK1 y se activa el influjo de calcio a través de los canales CNGC2 y CNGC4. BIK1 ocasiona la fosforilación del dominio C-terminal citosólico de CNGC4 y se activan los canales CNGC2-CNGC4 (Y. Lu & Tsuda, 2021; W. Tian et al., 2019) **(Figura 15)**. A su vez, estos canales son regulados por el receptor NLR del tipo CC RPS2 que provocan la muerte celular programada tras el reconocimiento de proteínas Avr. De esta forma, la señalización dependiente de NLR regula el influjo de calcio en la célula (Clough et al., 2000; Jurkowski et al., 2004; Y. Lu & Tsuda, 2021).

2)Señalización por ROS. Estas moléculas son muy importantes durante la respuesta de defensa de la planta ya que actúan en la señalización y también como un "arma directa" frente a los microorganismos patógenos (Ishiga & Ichinose, 2016; Y. Lu & Tsuda, 2021; J. Qi et al., 2017; M. A. Torres, 2010). En la planta *A. thaliana,* ciertos PRRs están relacionados con la NADPH oxidasa RBOHD que se localiza en la membrana plasmática. BIK1 es capaz de regular positivamente a RBOHD a través de la fosforilación de serinas (ser) en la región N-terminal (Ser39, Ser339 y Ser343). A su vez, las proteínas dependientes de calcio, CPK4, CPK5, CPK6 y CPK11, fosforilan otras serinas (Ser133, Ser148 y Ser163). Del mismo modo, BIK1 y CPKs fosforilan la serina 347 (Dubiella et al., 2013; Kadota et al., 2014; Y. Lu & Tsuda, 2021) **(Figura 15)**. Los MAMPs son capaces de desencadenar la fosforilación de los residuos de serina en RBOHD que es necesaria para la generación de ROS (Lu & Tsuda, 2021). Además, se ha descrito mediante un sistema inducible que la activación del receptor NLR del tipo CC RPS2, estimula la acción de RBOHD (Y. Lu & Tsuda, 2021; Ngou et al., 2020; M. Yuan, Jiang, et al., 2021).

3)Señalización por MAPKs. La señalización entre los complejos PRR-citoplasma-núcleo, se da a través de las cascadas de MAPKs y la fosforilación de las proteínas que la integran. En la planta *A. thaliana,* existen dos rutas MAPKs actúan paralelamente en la señalización de la respuesta de defensa de la planta: MAPKKK3/5-MKK4/5-MPK3-6 y MEKK1-MKK1/2-MPK4. Como respuesta a flg22, elf18, quitina y pep2, se produce la fosforilación de MAPKKK3 y MAPKKK5. La activación de los PRR de la familia RLCK VII producen la fosforilación de la serina 599 de MAPKKK5. A su vez, gracias a MKK4 se produce la activación de MPK3 y MPL6. Estas producen la resistencia a la enfermedad a través del reconocimiento de MAMPs **(Figura 15)**. Del mismo modo, los receptores de la familia RLCK VII producen la fosforilación de la serina 603 de MEKK1, que activa MPK4 a través de MKK1 y MKK2 y la inhibición de SUMM2 (Bi et al., 2018; Y. Lu & Tsuda, 2021). Además, se ha visto que el efector de *Pseudomona syringae* AvrRpt2, es reconocido por el

receptor NLR de tipo CC RPS2 que provoca la activación constante de la cascada de MAPKs (Y. Lu & Tsuda, 2021; Tsuda et al., 2013).

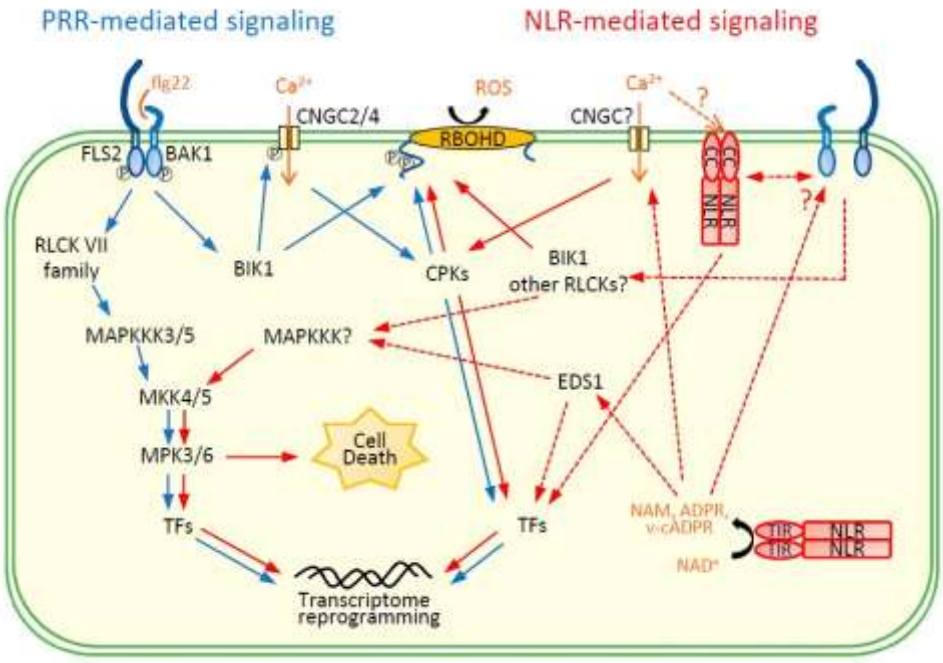


Figura 15. Modelo de interconexión de rutas de señalización de receptores PRR y NLR. Las flechas azules y rojas representan la señalización producida por los receptores PRRs y NLRs, respectivamente. Las flechas punteadas describen mecanismos que son hipotéticos. Por otro lado, hay mecanismos que no han sido demostrados experimentalmente y se representan con el símbolo (?). Esta figura ha sido extraída del estudio de Lu & Tsuda, 2021.

4)Señalización por rutas hormonales. Existen diferentes fitohormonas participan en la respuesta inmunitaria de la planta como el ácido salicílico (SA), etileno (ET) y el ácido jasmónico (JA). De manera general, la resistencia frente a patógenos biotróficos está medida por el SA, mientras que para patógenos necrotróficos, está regulada por JA (Glazebrook, 2005; Y. Peng et al., 2018). Ciertos estudios describen como interaccionan los receptores con las rutas hormonales. Se ha descrito que el aumento del etileno se produce a través de MPK3 y MPK6 tras desencadenarse la respuesta de defensa PTI (Liu & Zhang, 2004; Peng et al., 2018). Los receptores NLS como SCN1, RRP4, RPS2 participan en la resistencia a patógenos. Se ha visto como el SA colabora en este proceso (Nawrath & Métraux, 1999; Y. Peng et al., 2018; Van Der Biezen et al., 2002; Yuelin Zhang et al., 2003).

5)Señalización y factores de transcripción. Las respuestas de defensa PTI y ETI confluyen a través de los factores de transcripción SARD1 y CBP60g. Se observó como ambos factores de transcripción se inducían frente al tratamiento de flg22 y también frente a *P. syringae* que no era capaz de producir el sistema de secreción de tipo III. Del mismo modo ocurrió con la respuesta de defensa ETI, que favoreció la inducción de *SARD1* y *CBP60g* (Kong et al., 2016; Peng et al., 2018; Wang et al., 2009; Zhang et al., 2010). Además, se vio como la respuesta inmunitaria de la planta se veía activada por la expresión constitutiva de estos genes (Zhang et al., 2010; Peng et al., 2018). Por otro lado, SARD1 y CBP60g también participan en la regulación de genes que están relacionados con la ruta del SA (en la biosíntesis y la percepción hormonal) (Y. Peng et al., 2018; T. Sun et al., 2015). Finalmente, SARD1 y CBP60g juegan un papel en el control de la expresión de componentes de la respuesta de defensa PTI (BAK1, AGB1, BIJ1, MEKK1, MKK4, MPK3 y CPK4) y ETI (EDS1, PAD4, ADR1 y ADR-L1, ADR-L2) (Sun et al., 2015; Peng et al., 2018).

Los efectores en los hongos fitopatógenos

Las efectores son moléculas que son liberadas por microorganismos que utilizan para contrarrestar el sistema de supervivencia del huésped durante el proceso infectivo (Bent & Mackey, 2007; O' Connell & Panstruga, 2006; Win et al., 2012; Win et al., 2007). En el caso de los hongos patógenos de plantas, juegan un papel fundamental para la virulencia del hongo y la patogénesis (Bolton et al., 2008; Yoshida et al., 2009; Mosquera et al., 2009; Vargas et al., 2016). Estos pueden facilitar la penetración en el huésped, la absorción de nutrientes vitales para el desarrollo del hongo, camulfan las hifas del hongo u otros efectore, y producen la inhibición de la respuesta inmunitaria de la planta (respuestas PTI y ETI) (Khan et al., 2018; Landete, 2016; Pradhan et al., 2021; Stergiopoulos & de Wit, 2009; Vleeshouwers & Oliver, 2014). A menudo, los efectores suelen ser específicos de un linaje. Estos son definidos como aquellos que no tienen homología con ninguna otra proteína y que no tienen dominios conservados. Además, es posible que las especies de hongos más emparentadas, a veces no compartan homología (Riccardo Baroncelli et al., 2016; Fouché et al., 2018). Los efectores pueden clasificarse según su localización en: efectores apoplásticos (que se liberan al espacio extracelular de la planta) y efectores citoplasmáticos (que son transportados al interior celular) (Giraldo & Valent, 2013; Kamoun, 2006). Dentro del grupo de efectores apoplásticos se han descrito efectores que protegen la pared celular fúngica evitando su degradación; o bien, obstaculizan el reconocimiento del huésped gracias a la unión de los efectores a PAMPs o receptores PRR de la membrana plasmática; y otros, pueden suprimir la acción de las proteasas apoplásticas (De Jonge et al., 2010; Mueller et al., 2013; Uhse & Djamei, 2018; van den Burg et al., 2006; Wawra et al., 2016). A su vez, los efectores citoplasmáticos pueden transportarse a otros compartimentos subcelulares como el núcleo, nucleolo, cloroplastos, mitocondria, cuerpos del sistema de Golgi, microtúbulos y peroxisomas (Petre et al., 2015; Robin et al., 2018; Sperschneider et al., 2017; Uhse & Djamei, 2018; Vargas et al., 2016). El reconocimiento de la función que llevan a cabo los efectores, permite conocer de mejor forma el papel que juegan durante el desarrollo de las enfermedades, así como en el establecimiento de nuevas estrategias para combatir los hongos patógenos de plantas) (Kanja & Hammond-Kosack, 2020).

Dependiendo del estilo de vida, los hongos se han especializado en la liberación de efectores que cumplen diversas funciones. Los efectores de los hongos biotróficos juegan un papel importante inhibiendo el sistema inmunitario de la planta, favoreciendo su colonización. Por otro lado, los hongos necrótrofos estimulan la susceptibilidad de la planta a través de la muerte celular programada (Bolton et al., 2008; Meena & Samal, 2019; Vleeshouwers et al., 2008; G. Yang et al., 2018) **(Figura 16)**. Los hongos hemibiotróficos liberan distintos efectores según en qué fase de su estilo de vida se encuentren, biotrófico o necrotrófico (Gawehns et al., 2014; Khang et al., 2008; Zhang et al., 2015).

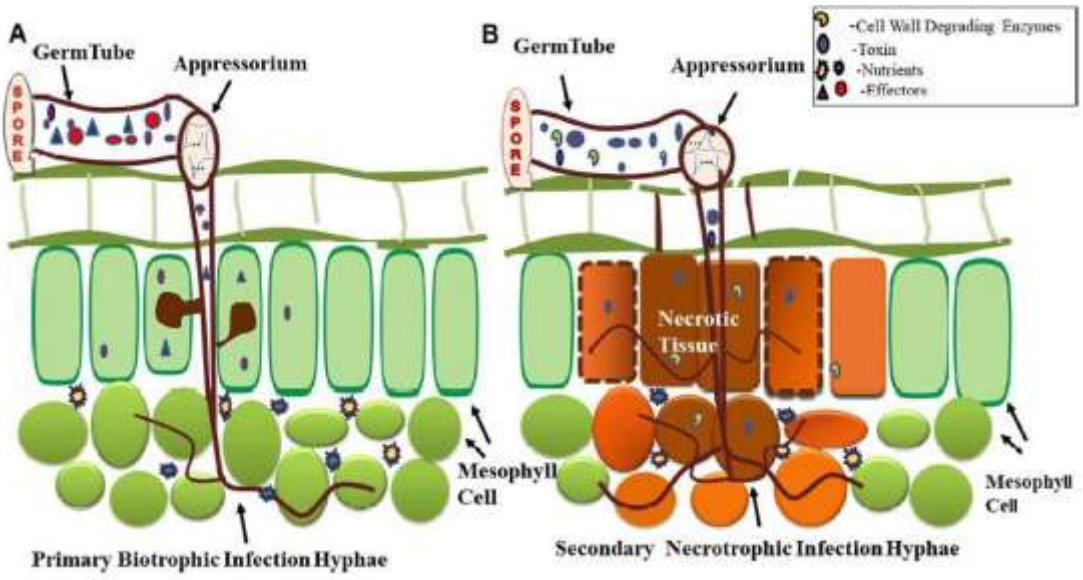


Figura 16. Los hongos fitopatógenos liberan efectores durante la colonización del huésped. A) Durante el estilo de vida biotrófico, el patógeno secreta efectores que mantienen vivas a las células y contrarrestan el sistema inmune de la planta. **B)** Durante el estilo de vida necrotrófico, los efectores secretados por el patógeno ayudan a producir la muerte celular programada del huésped. Además, utilizan una gran cantidad de toxinas y enzimas hidrolíticas que ocasionan la necrosis del tejido donde el hongo se desarrolla. Esta figura ha sido extraída del estudio de Pradhan et al., 2021.

Otra característica de los efectores descubierta recientemente es que pueden interaccionar con el microbioma de los huéspedes. La salud de la planta puede estar influenciada por su microbioma asociado. El microbioma puede actuar de manera decisiva inhibiendo el proceso infectivo, beneficiando de esta forma a la planta. A su vez, algunos efectores son capaces de afectar al microbioma del interior y del exterior de la planta cambiando su composición (Snelders et al., 2020).

Evolución de los efectores en hongos patógenos de plantas

Los hongos fitopatógenos cuentan con genes que codifican efectores que pueden vencer la resistencia de sus huéspedes con rapidez. Así, las variaciones genéticas que se dan en el genoma fomentan los procesos evolutivos que benefician al patógeno y pueden ser obtenidas por otras especies (Badet & Croll, 2020a; Fouché et al., 2018; Lo Presti et al., 2015). Se ha descrito que los genomas de los hongos filamentosos pueden tener mutaciones puntuales, o bien, presencia o ausencia de genes individuales, grupos de genes o incluso grandes segmentos de cromosomas (Badet & Croll, 2020a; Bertazzoni et al., 2018). Para analizar dichas variaciones, en la actualidad se están llevando a cabo estudios de pangenómica. Un pangenoma se define por el número total de genes individuales que tienen posiciones homólogas entre los genomas de la misma especie (Badet & Croll, 2020a; Zhaoen Yang et al., 2019). Un pangenoma también pueden definirse a través del conjunto de ortogrupos, que muestran las similitudes entre las secuencias de las proteínas. Estos pueden tener funciones parecidas entre los miembros del grupo. El estudio de los ortogrupos favorece la búsqueda de las reorganizaciones génicas que se producen en el genoma. Sin embargo, los genes ortólogos pueden no encontrarse en el mismo locus, o bien, se puede crear una redundancia funcional debido a la alta frecuencia de duplicaciones genéticas de dichos genes (Badet & Croll, 2020a; Golicz et al., 2016; W.-B. Jiao & Schneeberger, 2020; J.-M. Song et al., 2020). Además, los pangenomas permiten estudiar como los elementos

transponibles y las regiones no codificantes participan en las variaciones genéticas y en la adaptación (Badet & Croll, 2020).

Los hongos patógenos de plantas muestran conjuntos de efectores y genes asociados con la patogenicidad que pueden tener altas tasas de variación (Fouché et al., 2018, Badet & Croll, 2020). Los conjuntos de efectores pueden encontrarse en regiones accesorias del genoma (como los cromosomas accesorios) o bien, en regiones repetitivas de los cromosomas (Badet & Croll, 2020; Ma et al., 2010), que suelen estar asociadas una alta abundancia de elementos transponibles (Badet & Croll, 2020; Faino et al., 2016; Grandaubert et al., 2014; Ma et al., 2010; Peng et al., 2019) . Se definen como regiones de evolución rápida y se localizan en las áreas subteloméricas en las que a menudo se dan inserciones y deleciones de secuencias (Badet & Croll, 2020; Brown et al., 2010; Rehmeyer et al., 2006; Van Wyk et al., 2018; Wyatt et al., 2020). Por otro lado, aparecen regiones que evolucionan más lentamente, que tienen un bajo nivel de repeticiones y donde suelen encontrarse los genes de mantenimiento celular (Dong et al., 2015; Hastings et al., 2009; Raffaele et al., 2010; Raffaele & Kamoun, 2012; Torres et al., 2020). Gracias a que los genomas tienen este tipo de organización, se describió el modelo de "dos velocidades", que permite la evolución de los genomas de hongos fitopatógenos (Dong et al., 2015; Frantzeskakis et al., 2019; Raffaele & Kamoun, 2012; Torres et al., 2020). Las regiones ricas en repeticiones favorecen la generación de nichos de efectores que favorecen su adaptación (Torres et al., 2020). Sin embargo, no todos los efectores se encuentran en estas regiones que producen una evolución rápida (Fouché et al., 2018).

Los efectores tienen que evitar ser reconocidos por los receptores de las plantas. Esto ha posibilitado que se den interacciones gen a gen de manera intensiva que favorecen su evolución. A menudo, la patogenicidad puede estar regulada por genes individuales y el éxito del patógeno durante el proceso infectivo, puede verse determinado por la ganancia o pérdida de tan solo un gen efector (Badet & Croll, 2020; Liao et al., 2016). Así, el patógeno y el huésped pueden coevolucionar a través de una carrera de armamentos que permiten su adaptación (Badet & Croll, 2020; Sánchez-Vallet et al., 2018; Stukenbrock & McDonald, 2009). La localización de efectores en regiones que presentan altas variaciones, puede tener un papel clave en la evolución de las interacciones del huésped con el patógeno (Badet & Croll, 2020b; Dong et al., 2015; Fouché et al., 2018) **(Figura 17).**

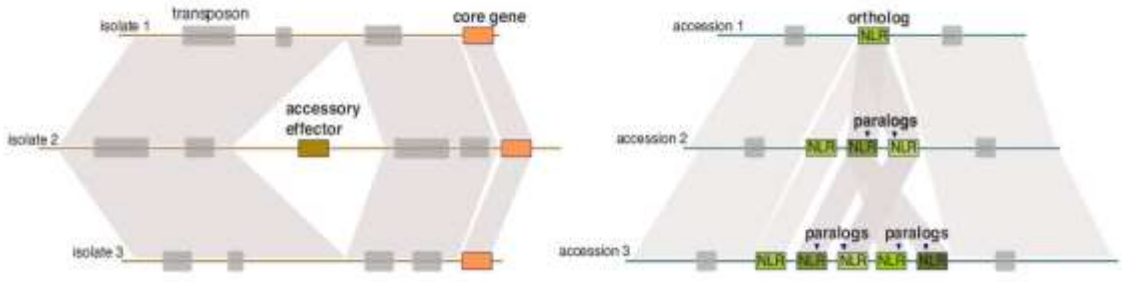


Figura 17. Evolución de los efectores y los receptores NLR en las interacciones huésped-patógeno. A la izquierda, las variaciones genéticas durante la evolución permiten la ganancia o pérdida de los efectores en los hongos patógenos. Los efectores suelen encontrarse en regiones donde la sintenia no está conservada entre los genomas. A menudo, aparecen en regiones ricas en elementos transponibles. A la derecha, los reordenamientos no homólogos producidos en la evolución, pueden ocasionar la aparición de genes parálogos que codifican receptores NLR. Esto permite su diversificación. Esta figura ha sido extraída del estudio de Badet & Croll, 2020.

El nacimiento de los efectores puede producirse a través de las duplicaciones genéticas que surgen como consecuencia de las recombinaciones homólogas de regiones que contienen elementos transponibles. A su vez, la copia generada por recombinación puede sufrir mutaciones de manera rápida, lo que favorece su neofuncionalización. Durante la evolución de la copia generada también puede adquirir una señal de secreción (Fouché et al., 2018; Poppe et al., 2015). Los efectores también pueden originarse *de novo.* Este mecanismo de nacimiento de genes implica que una secuencia de ADN no codificante, se transforme en un marco de lectura abierto para que la secuencia se vuelva funcional. Como resultado, se origina un nuevo gen (Fouché et al., 2018; McLysaght & Guerzoni, 2015; McLysaght & Hurst, 2016; Plissonneau et al., 2017) . De la misma forma, los efectores pueden obtenerse a partir de la transferencia horizontal de genes desde otros organismos como hongos y plantas (de Jonge et al., 2012; Friesen et al., 2006; Ma et al., 2010). Estos provocan cambios drásticos en el estilo de vida del hongo y durante su evolución (Fouché et al., 2018). Otra fuente de origen de genes es la hibridación génica. Esta puede producirse entre una especie parental y otra especie receptora que son sexualmente compatibles, mediante la introgresión del material genético y sucesivos retrocruzamientos entre ambas especies. La fase de hibridación es transitoria (Baack & Rieseberg, 2007; Fouché et al., 2018; Stukenbrock, 2016a). La hibridación génica es una de las fuentes más importantes para el nacimiento de genes y permite la adaptación de los hongos patógenos a nuevos huéspedes o ambientes (Fouché et al., 2018; Stukenbrock, 2016b). Por otro lado, la presión de selección intensa producida en las interacciones huésped-patógeno, favorecen la fijación de mutaciones no sinónimas que evitarían el reconocimiento de los efectores de los hongos patógenos por sus huéspedes (Fouché et al., 2018). A su vez, la desaparición de los efectores en los hongos patógenos puede originarse a través de las deleciones génicas, o mediante recombinaciones no homólogas que se producen en el locus del efector. Como resultado, el efector pierde su función (de Jonge et al., 2013; Faino et al., 2016; Fouché et al., 2018). Otros procesos por los que un efector puede perder su función son a través de la inserción de elementos transponibles en la región promotora o la región codificante del efector, silenciamiento epigenético, entre otros (Fouché et al., 2018).

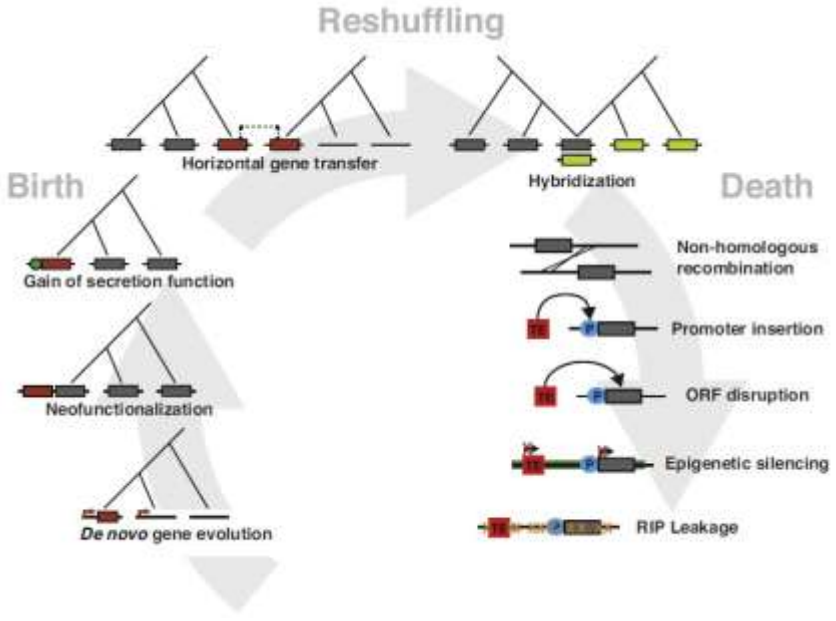


Figura 18. Nacimiento y pérdida de efectores en los hongos fitopatógenos. Los efectores pueden evolucionar *de novo,* a partir de la conversión de una región no codificante en un marco de lectura abierto; a través de las duplicaciones génicas y neofuncionalización; y también gracias a la adquisición de una señal de secreción. Los

efectores a su vez, emergen a través la transferencia horizontal génica o mediante procesos de hibridación. Los efectores pueden perder su función a través de las recombinaciones no homólogas producidas en el locus del efector; mediante la inserción de elementos transponibles en la región promotora o en la región codificante del gen, que permiten su alteración; mutaciones puntuales en la región genómica del gen; o bien, a través del silenciamiento epigenético. Esta figura ha sido extraída del estudio de Fouché et al., 2018.

Los efectores en los hongos fitopatógenos contrarrestan las defensas de la planta

Los efectores son secretados por los hongos patógenos hacia el apoplasto del tejido vegetal para contratacar las barreras de defensa física y química primarias de la planta. Desde aquí, pueden transportarse al interior celular donde interaccionan con componentes de la membrana plasmática, citosol y otros orgánulos celulares. Estos efectores facilitan el desarrollo del hongo en el huésped, evadiendo la respuesta de defensa de la planta (Tariqjaveed et al., 2021). Para esta sección de la introducción se ha utilizado la revisión más reciente de Tariqjaveed et al. (2021). Los efectores se encuentran agrupados según su lugar de actuación:

1) Efectores apoplásticos. La planta es capaz de liberar una variedad de proteasas y compuestos antifúngicos en el apoplasto que le ayudan a establecer una barrera física y química con la que combatir la infección ocasionada por el hongo patógeno. Por otro lado, el hongo es capaz de secretar efectores con los que superar dichas barreras (Tariqjaveed et al., 2021). Dentro de este grupo se encuentran las enzimas degradadoras de la pared celular (glucósido hidrolasas, glicosiltransferasas y pectín liasas) cuya función es la hidrólisis de la pared celular vegetal (Kubicek et al., 2014). *M. oryzae* libera celulasas y endo-β-1,4-xylanasas (de la familia de las glucósido hidrolasas), que favorecen la penetración del hongo y expansión (Nguyen et al., 2011; Van Vu et al., 2012). Otro ejemplo está representado por la cutinasa CgICUT1 de *C. gloeosporioides* que permite la rotura de la cutina y facilita la virulencia del hongo (Wang et al., 2017). La xilanasa RcXYN1 de *Rhizoctonia cerealis* fomenta la colonización del hongo en plantas de trigo a partir de la inducción de la muerte celular programada y la producción de ROS (Lu et al., 2020). Otros efectores se han especializado en contrarrestar la respuesta de defensa desencadenada por quitina de la planta. La quitina forma parte de las paredes celulares de los hongos y puede ser reconocida por los receptores PRR que tienen motivos LysM provocando la respuesta de defensa (Miya et al., 2007; Sanchez-Vallet et al., 2015). Los efectores pueden ayudar al hongo, bien, evitando el reconocimiento de los oligosacáridos de quitina por los receptores PRR con motivos LysM, o previniendo la acción de las quitinasas vegetales (Kombrink & Thomma, 2013; Sánchez-Vallet et al., 2013; Sanz-Martín et al., 2016a; Volk et al., 2019) . El efector Ecp6 de *Cladosporium fulvum,* es un efector que tiene dominios LysM que une la quitina, evitando que pueda ser identificada por la planta) (De Jonge et al., 2010; Sánchez-Vallet et al., 2013). Los efectores ChELP1 y ChELP2 de *C. higginsianum* son capaces de inhibir las MAPKs que transducen la respuesta de defensa desencadenadas por quitina en *A. thaliana* (Takahara et al., 2016). Por otro lado, las plantas secretan las cisteín proteasas del tipo papaína (PLCP) que son importantes para la respuesta inmunitaria (Liu et al., 2019; Misas Villamil et al., 2019). El efector Avr2 del hongo *C. fulvum* suprime varias PLCP que la planta necesita para la respuesta de defensa (van Esse et al., 2008). El efector Pit2 de *U. maydis* es capaz de inhibir la acción de CP1, CP2y XCP2, un grupo de cisteín proteasas del maíz (Mueller et al., 2013). Otros efectores pueden interaccionar con proteínas relacionadas con la patogénesis (PR), como SnToxA de *Stagonospora nodorum,* o SsCP1 de *Sclerotinia sclerotiorum* cuya diana es PR-1, que regula las defensas del huésped (Breen et al., 2016; Yang et al., 2018).

3) Efectores citoplasmáticos **(Figura 19)**. Este tipo de efectores interfieren en numerosas funciones. El efector AvrPiz-t de *M. oryzae* se une al transportador de potasio OsAKT1 y la proteína quinasa citosólica OsCIPK23 en las plantas de arroz. Se produce la inhibición de la

señalización de la respuesta de defensa mediada por potasio (X. Shi et al., 2018). Algunos efectores pueden intervenir en la respuesta de señalización de las fitohormonas. El efector SnTox3 de *S. nodorum* estimula la actividad de la señalización de la ruta de ET, inhibiendo la señalización por SA y la generación de ROS, así como reduciendo las citoquininas activas. Como resultado de esta compleja interacción, el trigo se vuelve susceptible al hongo y puede infectarlo (Veselova et al., 2021). En cuanto al estallido de ROS, el efector AVR-Pii de *M. oryzae* es capaz de suprimir en condiciones *in vitro* dicho estallido y la actividad de la OsNADP-ME2 en el arroz. Esta enzima es importante para el incremento de ROS en la planta (Dangol et al., 2019; X. Shi et al., 2018; R. Singh et al., 2016). En los últimos años se han descubierto efectores que pueden degradar las moléculas de ARN y están implicados en la patogenicidad del hongo. Así, por ejemplo, el efector CSEP0064/BEC1054 del hongo biotrófico *Blumeria graminis* es una proteína del tipo ARNasa. Este efector puede evitar la degradación del RNA ribosomal del huésped, favoreciendo que las células vegetales permanezcan vivas y así poder utilizarlas para alimentarse (Pennington et al., 2019). Otros efectores pueden afectar a la estabilidad de las proteínas del huésped, como es el caso del efector Tin2 de *U. maydis.* Este efector permite la preservación de la actividad quinasa de la proteína ZmTTK1 de plantas de maíz, evitando su degradación por la vía ubiquitina-proteosoma. Tin2 es necesaria para favorecer la patogénesis de *U. maydis* sobre el maíz, reorientando la ruta de la síntesis de la lignina hacia la ruta de síntesis de las antocianinas (S. Tanaka et al., 2014).

3)Efectores nucleares **(Figura 19)**. Estos efectores se caracterizan por conservar una secuencia de localización nuclear en su estructura, por lo que son transportados al núcleo de la planta (Kemen et al., 2005; Klosterman et al., 2011; Petre et al., 2015; Robin et al., 2018; Vargas et al., 2016). Los efectores nucleares son capaces de interferir en la respuesta inmunitaria de planta y otros proceso celulares uniéndose a factores transcripcionales o proteínas del núcleo o nucleolo, reprogramando la expresión numerosos genes (Qi et al., 2018; Qin et al., 2018). Los efectores VdSCP7 y VdSCP41 de la especie *Verticillium dahliae* tienen como diana los factores de transcripción CBP60g y SARD1, que participan en la biosíntesis del SA. La interacción del efector con estar proteínas permite la interferencia de la respuesta de defensa de la plantas de tabaco frente al hongo (Qin et al., 2018; Zhang et al., 2017a). El efector PpEC23 de *Phakopsora parchyrhizi* puede relacionarse con el factor de transcripción GmSPL12l, inhibiendo la respuesta de defensa de plantas de soja (Qi et al., 2016). Los efectores MoHTR1 y MoHTR2 de *M. oryzae,* son capaces de unirse a promotores de genes que se encuentran relacionados con la respuesta inmunitaria del arroz, interfiriendo en dicha respuesta (Kim et al., 2020).

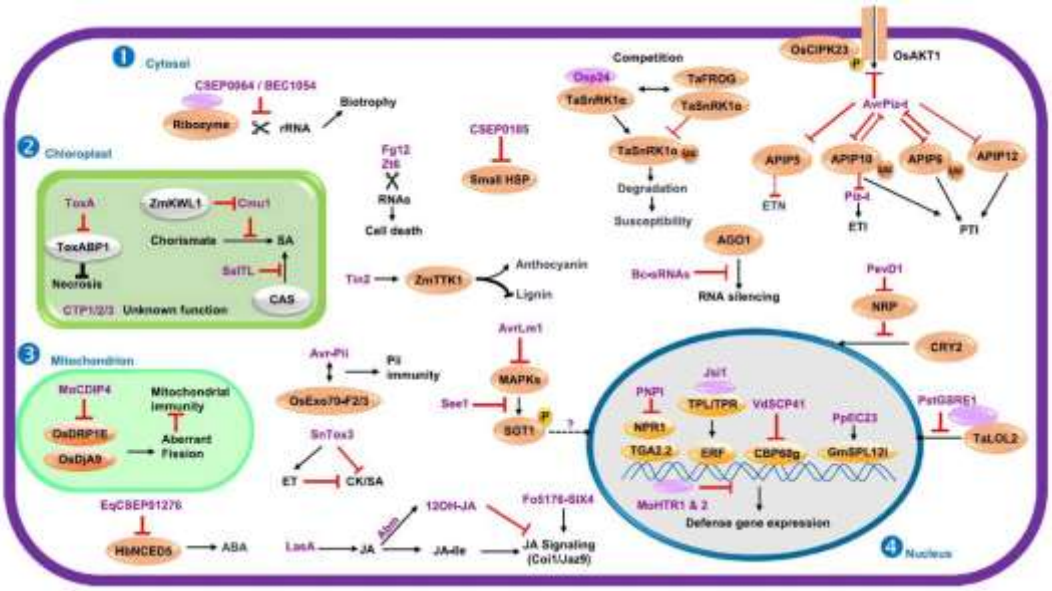


Figura 19. Modo de acción de algunos de los efectores intracelulares descritos en esta introducción. Los efectores pueden ser transportados desde el apoplasto al interior de la célula. Allí pueden actuar en el citoplasma o a su vez, trasladarse a otros orgánulos como los cloroplastos, las mitocondrias o el núcleo. Esta figura ha sido extraída del estudio de Tariqjaveed et al., 2021.

4)Efectores cloroplásticos **(Figura 19)**. Los cloroplastos participar en la respuesta de defensa ya que producen importantes componentes inmunitarios como SA o ROS, entre otros) (de Torres Zabala et al., 2015; Saijo & Loo, 2020; Serrano et al., 2016). Se encuentran algunos ejemplos como los efectores ToxA y ToxB que son toxinas liberadas por *P. tritici-repentis* que participan en la patogénesis del hongo. Se ha visto como ToxA tiene como diana a la proteína ToxABP1 en el cloroplasto de plantas de trigo (Manning et al., 2009, 2010). El efector Pst_12806 de *P. striiformis* f. sp. *tritici* participa inhibiendo la respuesta de defensa en el cloroplasto. Es capaz de unirse a TaISP, un miembro del complejo citocromo b6-f. Este efector es capaz de disminuir la actividad fotosintética, la cadena de transporte de electrones y la generación de ROS.

5)Efectores mitocondriales **(Figura 19)**. La mitocondria también juega un papel importante para la inmunidad de la planta durante la señalización mediada por ROS y SA, entre otros (Colombatti et al., 2014). El efector Avr-Pita de *M. oryzae* tiene como diana a la proteína OsCOX11 que participa en el metabolismo de ROS de la mitocondria en el arroz. El efector es capaz de incrementar la actividad de OsCOX11 y como consecuencia, disminuye la producción de ROS (Colombatti et al., 2014). Otro efector de la misma especie, MoCDIP4, se une al complejo OsDjA9-OsDRP1E de la mitocondria cuyo resultado, produce la inhibición de la respuesta de defensa del arroz. Además, el efector provoca un crecimiento anormal de la mitocondria y esta se alarga (G. Xu et al., 2020).

Efectores en *Colletotrichum graminicola*

Durante los últimos años se han incrementado el número de investigaciones que tienen por objetivo estudiar los efectores del hongo que ocasiona la antracnosis del maíz. Sin embargo, todavía son pocos **(Tabla 1)**.

Gen	Descripción	Función	Mutante	Virulencia	Presencia	Localización	Referencia
CgEP1	Proteína efectora	Interacción con ADN	Sí	Reducida	Específico de *Colletotrichum*	N	Vargas *et al*., 2016
Cgfl/CgEP2	Metaloproteasa	Control de la señal de quitina	Sí	Reducida	Conservado en los hongos	SD	Sanz-Martín *et al*., 2016a
CgCPLS	Subtilisina	Proteasa	Sí	Hipervirulencia	Específico de *Colletotrichum* y *Diaporthe*	SD	Sanz-Martín, 2016
CgCLU5a	Grupo CLU5a/d	Efector putativo	Sí	Reducida	Conservado en los hongos	SD	Eisermann *et al*., 2019
CgCLU5d	Grupo CLU5a/d	Efector putativo	Sí	Reducida	Conservado en los hongos	SD	Eisermann et al., 2019
CgCFEM6	Proteína que contiene dominios CFEM	Efector putativo	No	Inhibición de la muerte celular programada parcialmente (*)	Conservado en los hongos	N (*)	Gong *et al*., 2020
CgCFEM7		Efector putativo	No	Inhibición de la muerte celular programada por completo (*)	Conservado en los hongos	MP (*)	Gong *et al*., 2020
CgCFEM8		Efector putativo	No		Específico de *Colletotrichum*	N (*)	Gong *et al*., 2020
CgCFEM9		Efector putativo	No		Conservado en los hongos	MP, CC (*)	Gong *et al*., 2020
CgCFEM15		Efector putativo	No		Conservado en los hongos	MP, C (*)	Gong *et al*., 2020
CgCEC3	Proteína efectora	Efector	No	Muerte celular débil, expansión nuclear (**)	Específico de *Colletotrichum*	N (**)	Tsushima *et al*., 2021

Tabla 1. Efectores estudiados en la actualidad en la especie *Colletotrichum graminicola.* Nombre del gen. Se añade una pequeña descripción y la función que desempeñan. Se indica si el efector ha sido caracterizado a través de la deleción del gen. La virulencia que presentaban tras la infección. (*) (**), la virulencia de estos efectores fue estudiada expresando la secuencia del gen de manera heteróloga en plantas de tabaco (*Nicotiana benthamiana*). Además (**), la respuesta podría ser débil debido a que la proteína expresada en las plantas de tabaco estuviera plegada de manera incorrecta o no fuera funcional. La columna de presencia indica los grupos de hongos donde se conserva el efector. Localización del efector en el huésped: N = nuclear; MP = membrana plasmática; C = citosol; CC = cuerpos citoplasmáticos; y SD = sin datos.

El primer efector que se caracterizó en la especie *C. graminicola* fue CgEP1 (Vargas et al., 2016). Este efector se localiza en el núcleo de las células del maíz durante el proceso infectivo. CgEP1 es el único efector nuclear descrito en la actualidad en *C. graminicola.* Análisis filogenéticos demostraron que CgEP1 provenía de una duplicación genética que se dio en un ancestro del género *Colletotrichum* que infectaba plantas monocotiledóneas. Como resultado de la duplicación se formaron *CgEP1* y su parálogo, *GLRG_09337.* Además, se encontraron homólogos de CgEP1 en otras especies del género *Colletotrichum* por lo que es específico de este género. CgEP1 es un efector que estuvo sometido a presión de selección positiva durante la evolución, por lo que posibilitó la aparición de tres alelos del efector en diferentes estirpes de *C. graminicola,* que variaban en su estructura: desde cuatro a seis repeticiones intragénicas en

tándem. Análisis del perfil de expresión de *CgEP1* revelaron que se expresaba durante las etapas tempranas de la infección de hojas de maíz con *C. graminicola.* Posteriormente se demostró su participación en la virulencia de *C. graminicola* durante la infección de los tallos, hojas y raíces del maíz. Gracias a diversos estudios genéticos, moleculares y bioquímicos se concluyó que CgEP1 jugaba un papel importante en la colonización del maíz y el desarrollo de la antracnosis. Dado que CgEP1 se localizaba en el núcleo, podría estar regulando procesos que son clave para la respuesta de defensa de la planta contra *C. graminicola* (Vargas et al., 2016). Adicionalmente, en el trabajo de Vargas et al. (2016) también fueron identificados 27 efectores putativos que podrían tener localización nuclear (dos de los cuales se han sido caracterizados en esta tesis doctoral).

El segundo efector descrito en *C. graminicola* fue CgFl que codifica una fungalisina metaloproteasa (Sanz-Martín et al., 2016a). Este efector está relacionado con la degradación de enzimas quitinasas de la planta. La acción de estas enzimas puede degradar la quitina de la pared celular del hongo y activar la respuesta de defensa desencadenada por quitinas en la planta (de Jonge et al., 2011). Los análisis filogenéticos demostraron que CgFl se encuentra conservado en hongos de la clase Sordariomycetes, debido a su importante función. Gracias a experimentos de qRT-PCR se observó que CgFl se expresaba en la etapa biotrófica durante las infecciones de hojas de maíz. Los ensayos de patogenicidad llevados a cabo determinaron que CgFl participaba en la virulencia de *C. graminicola* en las hojas y raíces del maíz (Sanz-Martín et al., 2016a).

El tercer efector que se caracterizó en el hongo *C. graminicola* fue CPLS, que codifica una subtilisina que es una serín-proteasa (Sanz-Martín, 2016). De manera interesante, los estudios filogenéticos demostraron que CPLS provenía de un evento de transferencia horizontal génica que se produjo desde la planta al hongo. CPLS se encuentra conservado en el género *Colletotrichum* y también en *Diaporthe.* El análisis del perfil transcripcional de CPLS determinó que se expresaba durante las etapas tempranas de la infección de hojas de maíz (etapa biotrófica). Otra sorpresa que se observó fue que la deleción del gen *CPLS* producía un fenotipo de hipervirulencia en plantas infectadas con la estirpe mutante de este gen. Además, la deleción de *CPLS* también producía una reducción de la biomasa fúngica durante la infección. Estos resultados sugirieron que CPLS participaba en la disminución de la proliferación de *C. graminicola* durante el desarrollo de la infección en el maíz. De esta forma, el hongo puede controlar su crecimiento para evitar que la planta active la muerte celular (Sanz-Martín, 2016).

El genoma de *C. graminicola* tiene un grupo formado por 32 genes donde se encuentran *CLU5a* y *CLU5d* que codifican dos efectores putativos (Eisermann et al., 2019). Análisis filogenéticos revelaron que CLU5a y CLU5d estaban conservados en el género *Colletotrichum* y otras especies de la clase Sordariomycetes. Los ensayos de patogenicidad en hojas y tallos de maíz demostraron que ambos participaban en la virulencia de *C. graminicola.* Además, CLU5a y CLU5d son necesarios para que los apresorios puedan penetrar las células epidérmicas de la planta. De manera individual, CLU5a también podría jugar un papel en la función de la pared celular del hongo, fenómeno que se sugería tras realizar experimentos de frecuencia de la citorrisis. Las proteína CLU5a y CLU5d son consideradas como efectores putativos ya que son codificadas por genes que se expresan antes de la penetración y también durante el crecimiento vegetativo del hongo, por lo que estas proteínas no tendrían un papel de efector como función primaria (Eisermann et al., 2019).

El hongo *C. graminicola* secreta proteínas en cuya estructura tienen dominios CFEM que juegan un papel importante en la patogénesis del hongo (Gong et al., 2020). Análisis filogenéticos permitieron identificar 24 proteínas CFEM en el genoma de *C. graminicola* (CgCFEM1-24). Se realizaron alineamientos de las secuencias de los dominios CFEM cuyos resultados demostraron que se encontraban conservados en las proteínas CFEM. Se seleccionaron 10 proteínas que tenían un péptido señal que fueron consideradas como efectores putativos. Gracias a la expresión heteróloga y transitoria de las secuencias de los genes *CFEM* en plantas de tabaco, se predijo la localización subcelular. Los efectores putativos se encontraban en el núcleo, membrana plasmática y citoplasma. Por otro lado, se seleccionaron cinco de los diez efectores putativos, CgCFEM6, CgCFEM7, CgCFEM8, CgCFEM9 y CgCFEM15 para analizar si eran capaces de suprimir la muerte celular programada en las plantas de tabaco. Todos los efectores putativos suprimían dicha respuesta (aunque CgCFEM6 lo hizo de manera parcial). Se concluyó finalmente que estos efectores putativos podían tener un papel importante en la patogénesis del hongo Análisis filogenéticos posteriores revelaron que varios de estos efectores se encuentran conservados en los hongos, aunque CgCFEM8 era específico del género *Colletotrichum* (Gong et al., 2020).

El efector CEC3, es un "efector central" que se conserva en el género *Colletotrichum* (Tsushima et al., 2021). Para determinar el papel que podría jugar CEC3 en *Colletotrichum,* se expresaron de manera heteróloga y transitoria las secuencia de *CEC3* de cuatro especies de *Colletotrichum* en plantas de tabaco. Dichos genes pertenecían a las especies *C. higginsianum* (*ChCEC3*), *C. orbiculare* (*CoCEC3-1* y *CoCEC3-2*), *C. fructicola* (*CfCEC3-1* y *CfCEC-2*) y *C. graminicola* (*CgCEC3*) que son capaces de infectar diferentes huéspedes. En todos los casos se observó la expansión nuclear y la muerte celular programada en las plantas de tabaco. Sin embargo, el efector CgCEC3 produjo una respuesta débil. Según los autores del artículo, la proteína podría estar mal plegada o no ser funcional debido a que el gen se expresó en un sistema heterólogo. Finalmente se concluyó que los efectores que se encuentran conservados como CEC3 podrían tener un papel importante en las infecciones en un gran rango de huéspedes. Además, la función de CEC3 se conservaba en diferentes especies del género *Colletotrichum* (Tsushima et al., 2021).

HIPÓTESIS Y OBJETIVOS

HIPÓTESIS Y OBJETIVOS

A) El gen *CgEP3* de *Colletotrichum graminicola* codifica un efector que es específico de esta especie y está implicado en la virulencia.

- Objetivo 1A. Estudiar la evolución de *CgEP3* mediante análisis filogenéticos y de sintenia.

- Objetivo 2A. Predecir la localización subcelular de CgEP3 a través de herramientas *in silico* y ensayos experimentales.

- Objetivo 3A. Analizar el perfil de expresión de *CgEP3* mediante diferentes aproximaciones experimentales.

- Objetivo 4A. Generar transformantes mutantes nulos, complementados, y de expresión constitutiva del gen *CgEP3* y examinar su papel durante el crecimiento y desarrollo del hongo, así como durante el proceso infectivo en hojas y tallos de maíz.

- Objetivo 5A. Realizar análisis transcriptómicos a través de RNA-Seq y examinar los genes diferencialmente expresados de la planta que podían estar suprimidos o inducidos por la acción del efector durante la infección temprana de las hojas de maíz.

B) El gen *CgEP6* codifica una proteína efectora rica en prolinas que es conservada en las especies del género *Colletotrichum* y juega un papel importante en la virulencia.

- Objetivo 1B. Estudiar la evolución de *CgEP6* en el género *Colletotrichum* a través de análisis filogenéticos y sinténicos.

- Objetivo 2B. Realizar una predicción *in silico* y experimental de la localización subcelular de CgEP6.

- Objetivo 3B. Examinar el perfil de expresión de *CgEP6.*

- Objetivo 4B. Obtener transformantes mutantes nulos de *CgEP6* y estudiar su implicación durante la infección de hojas de maíz y en el crecimiento en diferentes medios de cultivo.

C) Los hongos aislados a partir de muestras infectadas de tallos de maíz de campos de Bosnia y Herzegovina, pertenecen a la especie *C. graminicola.*

- Objetivo 1C. Aislar e identificar estirpes de hongos desde tallos infectados de maíz.

- Objetivo 2C. Caracterizar mediante análisis filogenéticos las estirpes aisladas y comprobar los postulados de Koch a través de infecciones de hojas de maíz.

CHAPTER I:

CgEP3, a small species-specific effector from *Colletotrichum graminicola* that targets the host nucleus and aids in virulence at the early stages of infection.

ABSTRACT

The phytopathogenic fungus *Colletotrichum graminicola* causes a disease known as maize anthracnose. Leaf blight and stalk rot are the main symptoms in the field. The fungus can release effector proteins that allow it to improve host colonization through either manipulating plant cells or protecting the fungus from plant immunity responses. Using a previous genome-wide analysis of *C. graminicola*, we identified an effector candidate, *C. graminicola* effector protein 3 (CgEP3). It is a novel pre-penetration effector specific to *C. graminicola* localized to plant's nucleus and cytoplasm. This effector candidate has been predicted to be a secreted protein whose structure contains a partial nucleoside-phosphorylase super family-like domain (NP-like). Phylogenetic analysis revealed that *CgEP3* could have evolved from an ancestral gene duplication and diverged to encode effector proteins whose ancient function was to serve as a nucleoside-phosphorylase (NP). Furthermore, synteny analysis showed that the *CgEP3* genomic region could have evolved quickly, possibly as a result of ectopic recombination between different parts of the genome. We propose that CgEP3 plays a role in virulence during the infection of *C. graminicola* in maize at the early stages of infection and can help the fungi penetrate the plant epidermis. CgEP3 could help counteract the preinvasion defense mechanisms of maize, and thus, colonization could be carried out with less effort. To the best of our knowledge, this is the first species-specific effector described in *C. graminicola.*

INTRODUCTION

The genus *Colletotrichum* contains numerous plant pathogens that can cause important diseases that greatly impact agriculture (Bhunjun et al., 2021; Dean et al., 2012). These species cause anthracnosis and rot in post-harvested fruits, which result in major losses in high-value crops worldwide (Bhunjun et al., 2021; Phoulivong et al., 2010). *Colletotrichum graminicola* (Ces.) Wills., is one of the most characterized species that causes maize anthracnose disease in the field, whose main symptoms are leaf blight and stalk rot (Gong et al., 2020; Schliebner et al., 2014; Torres et al., 2016; Wang et al., 2016). It has a hemibiotrophic lifestyle and causes disease through three recognizable phases: appressoria formation, biotrophy, and necrotrophy (Gong et al., 2020; M. F. Torres et al., 2016).

Fungal pathogens need to overcome several plant defense systems to establish a compatible interaction (Eisermann et al., 2019; J. D. G. Jones & Dangl, 2006). Plants have developed an immune system with two layers that can detect and cope with a diversity of pathogens (Boller & He, 2009; J. D. G. Jones & Dangl, 2006; M. Yuan, Ngou, et al., 2021; Zhou & Zhang, 2020). The first layer is triggered by the recognition of pathogen-associated or damage-associated molecular patterns by pattern-recognition receptors localized on plant cell surfaces, a response known as pattern-triggered immunity (PTI) (M. Yuan, Ngou, et al., 2021), which plays an important role in avoiding pathogen invasion (Melotto et al., 2006; M. Yuan, Ngou, et al., 2021; C. Zipfel et al., 2004). Fungi can release effectors and other virulence-associated molecules to improve invasion and proliferation in the host. These factors enable the suppression of plant immunity. The plant can also activate a second immune response layer to combat the virulence of the pathogen called effector-triggered immunity. It is triggered by the recognition of effectors directly or indirectly by leucine-rich receptors (J. D. G. Jones & Dangl, 2006; M. Yuan, Ngou, et al., 2021). The immune response PTI and ETI are related through the crosstalk of the receptors and allows the maximum efficiency of the defense response of the plant (Bjornson & Zipfel, 2021).

Effector proteins are secreted by pathogenic fungi to promote host cell colonization either through interference or manipulation, as well as to protect the fungi from plant defense. These mechanisms can be diverse, including altering metabolism or hormone homeostasis, masking pathogen presence, or inducing necrosis. (Cook et al., 2015; Fouché et al., 2018). Effectors display a high range of genetic diversity because of the arms race between pathogens and hosts. They are classified depending on their location and include apoplastic effectors (secreted outside of host cells), cytoplasmic effectors (translocated into the cytoplasm of plant cells), and nuclear effectors (translocated inside the nucleus). Nuclear effectors have been studied in different organisms, including bacteria, oomycetes, and fungal pathogens (S. Kim et al., 2020). Effectors such as transcription activator-like from bacteria or *Phytophthora* crinkler from oomycetes, can suppress plant immunity either by inducing susceptibility or by manipulating the expression of host genes (S. Kim et al., 2020; T. Song et al., 2015; B. Yang et al., 2006). Several examples of fungal pathogens with nuclear effectors have been found in the Ascomycetes: *Magnaporthe oryzae* MoHTR1 and MoHTR2, *Verticillium dahliae* VdSCP41, and *Blumeria graminis* f. sp. *hordei* CSEP0064/BEC1054; and in the Basidiomycetes: *Ustilago maydis* See1, *Puccinia striiformis* f. sp. *tritici* PstGSRE1, *Uromyces fabae* UfRTP1, and *Melampsora larici-populina* MLHP124017 (de Guillen et al., 2019; Figueroa et al., 2021; Kemen et al., 2005; S. Kim et al., 2020; Pennington et al., 2019; Petre et al., 2015; T. Qi et al., 2019; Junhua Qin et al., 2019; Redkar et al., 2015). So far, few fungal nuclear effectors have been described in *C. graminicola.* CgEP1, which binds to host DNA and plays an important role during the biotrophic stage, when it interferes with the transcription of numerous maize genes. It is necessary for anthracnose disease development in the leaves, stalks, and roots (Vargas et al., 2016). Other nuclear effectors have been described, including CgCFEM6 and CgCFEM8, which suppress partial or complete cell death, respectively (Gong et al., 2020), and CgCEC3, which can induce a weaker defense response and nuclear expansion (Tsushima et al., 2021). However, additional analysis is needed to confirm the effector localization into the host nucleus during infection and whether effectors can control the expression of host genes related to immunity (S. Kim et al., 2020).

Previously, a genome-wide analysis of *C. graminicola* was carried out to look for candidate effectors that were predicted to be secreted and have nuclear localization (Vargas et al., 2016). We characterized a novel candidate effector (CgEP3) that participates in virulence during the early stages of maize infection and helps the fungus penetrate host cells. CgEP3 counteracts maize-preinvasion defense and prepares the cells for colonization with minimal resistance. *In silico* and experimental analyses showed that CgEP3 is a secreted protein with nucleo-cytoplasmic subcellular localization. Additionally, CgEP3 was predicted to contain a partial nucleoside phosphorylase domain. Phylogenetic analysis showed that *CgEP3* evolved from an ancestral gene duplication and diverged to encode an effector protein whose ancient functions was as nucleoside phosphorylase. Furthermore, synteny analysis showed that the *CgEP3* genomic region evolved quickly, which could be a result of ectopic recombination between different parts of the genome. CgEP3 was the first species-specific effector to be described in *C. graminicola.*

RESULTS

CgEP3 encodes a small effector

Vargas et al. (2016) identified 164 candidate effectors in *C. graminicola* which were predicted to be secreted and localized to the nucleus. We selected GLRG_00879 (*C. graminicola* effector protein 3, CgEP3) for further characterization. The gene structure of *CgEP3* has a 5' untranslated region (UTR) of 528 bp divided by an intron of 68 bp, a small coding sequence (CDS) of 222 bp that has one exon, and a 3' UTR of 962 bp **(Figure 20A)**. *CgEP3* encodes a protein 73-amino-acids long. Using InterProScan (Quevillon et al., 2005), we determined that the N-terminal region contains a signal peptide (SP) (22 amino acids in length) that overlaps with a nucleoside phosphorylase super family like-domain (IPR035994) (NP-like) **(Figure 20B)**. Because CgEP3 has an SP, we considered it to be a secreted protein. In addition, we believe that the NP-like domain is partial because it has a length of 64 amino acids. When we compared CgEP3 with the most similar ten NP proteins in the genus *Colletotrichum*, we found that the average length of the NP domain in those proteins was 290 amino acids (data not shown). The typical NP domain was larger than the NP-like domain of CgEP3, suggesting that the NP-like domain of CgEP3 was partial. To study the tertiary structure of CgEP3, we used a hypothetical three-dimensional model structure based on the homology modeling method using proteins found in public databases **(Figure 20B and Supplementary Figure 1)**. It has two alpha helices and three beta strands.

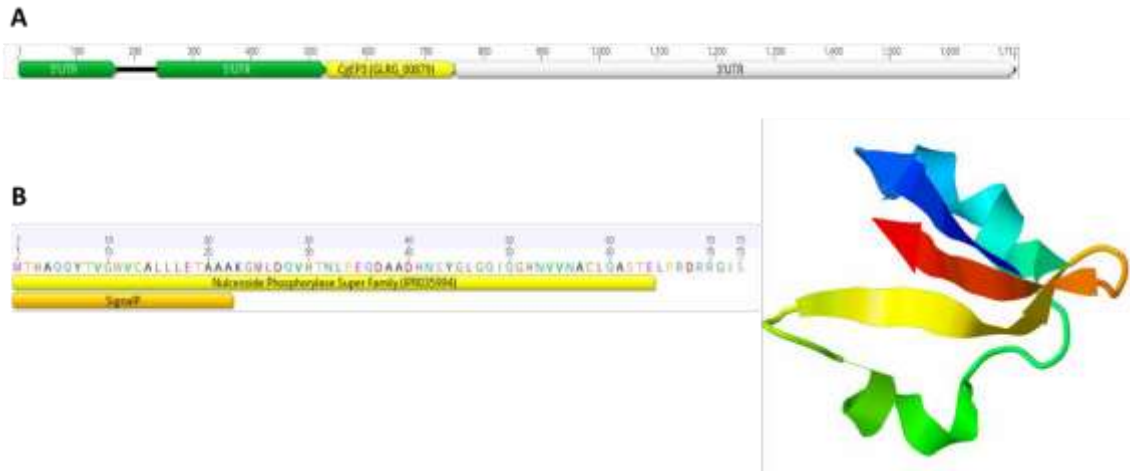


Figure 20. **CgEP3 is a small effector. A)** Genomic structure of *CgEP3* (*GLRG_00879*): The 5' UTR (green) is 528 bp long and is divided by an intron 68 bp long; the CDS has one exon and is 222 bp long (yellow); and the 3' UTR is 962 bp long (grey). **B)** On the left, *CgEP3* encodes a protein with 73 amino acids. We predicted that it contains a signal peptide which overlaps with a nucleoside phosphorylase super family-like domain (IPR035994). This domain is partial. On the right, a three-dimensional model structure based on the homology modeling method is shown. It has two alpha helices and three beta strands in its structure.

CgEP3 gene has a new structure

We determined that the original gene structure prediction by O'Connell et al. (2012) was not correct. The predicted gene structure of *GLRG_00879* is 446 bp in length and includes two exons and one intron. During reverse transcription-polymerase chain reaction (RT-PCR) experiments, we obtained a product that did not correspond with the expected length but aligned with the predicted intron **(Figure 21A)**. We then decided to investigate the *GLRG_00879* gene structure. Based on RNA-Seq data from ungerminated conidia samples of *C. graminicola* (shared by Dr. Stephan Wirsel, University of Martin Luther of Halle-Wittenberg, Germany) (unpublished data),

we were able to obtain evidence of a putative RNA transcript of *CgEP3* **(Figure 21B).** Next, we designed an experiment that consisted of multiple overlapping RT-PCRs that covered the *GLRG_00879* locus. The results provided evidence of a new putative transcript of *CgEP3,* which is longer than that described in the literature. We confirmed that the 5' UTR has an intron in its structure. We also discovered a transcribed region upstream of the *CgEP3*. Finally, we examined open reading frames in the transcript sequence **(Figure 21C)**. We described a new putative CDS structure of *CgEP3* that consists of one exon of 222 bp, which is smaller than that previously reported.

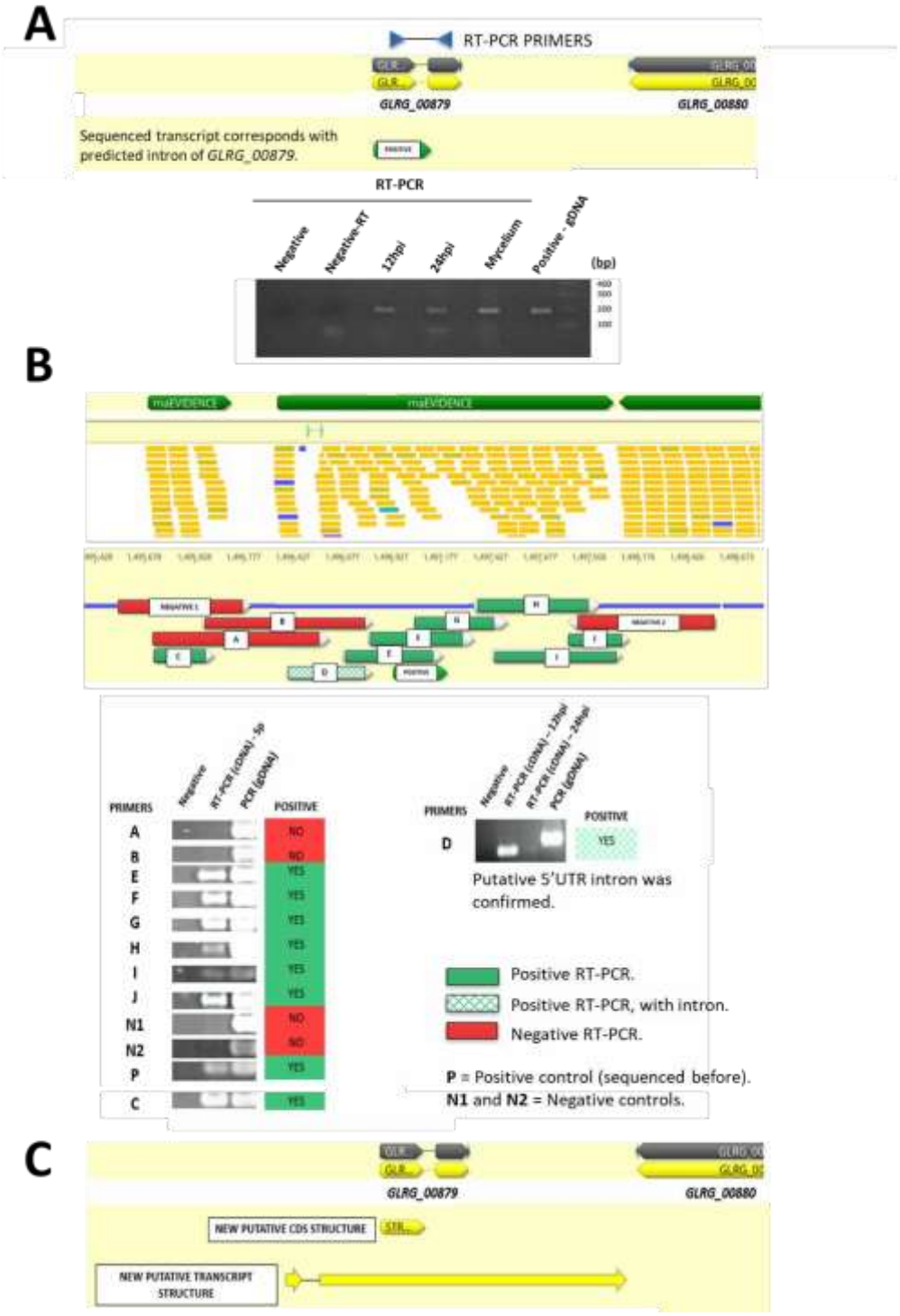


Figure 21. The *CgEP3* gene structure does not correspond with the predicted gene model. A) During RT-PCR experiments in the lab, we discovered that samples from maize leaves infected with *C. graminicola* at 12 and 24 h post-inoculation (hpi) and mycelium growing *in vitro* amplified a product that did not correspond with the predicted size. RT-PCR primers were designed to target the area between two exons, meaning that RT-PCR products should be smaller than those amplified from genomic DNA. We sequenced this product to confirm its identify and whether it could align with the intron of *GLRG_00879*. As controls, we used: water (negative PCR), negative RT-PCR (negative RT reaction), and genomic DNA (positive PCR). **B)** Based on RNA-seq evidence of conidia samples of *C. graminicola,* we designed an experiment that consisted of multiple overlapped RT-PCRs which covered *GLRG_00879* locus represented with letters (A-J, Negative 1 (N1), 2 (N2), and Positive(P)). Results showed positive RT-PCRs in green (D, E, F, G, H, I, and J) which amplified *CgEP3* transcripts. The results also corroborated that 5' UTR has an intron in its structure (D). Negative RT-PCRs appeared in red (N1, A, B and N2), delimitating the gene transcript. A transcribed region upstream of *CgEP3* was also discovered that was not described before (C). As positive control of RT-PCRs, we used genomic DNA and, for negative control, water. **C)** Putative *CgEP3* gene transcript and CDS. The transcript was longer than the previously described structure. In the same way, the CDS is smaller, and it does not have any intron.

CgEP3 is specific to *C. graminicola*

To study CgEP3 evolution, we performed a preliminary BLASTP search (Agarwala et al., 2016; Altschul et al., 1990; Boratyn et al., 2013) with the CgEP3 protein sequence. The results showed that CgEP3 had low similarity with proteins of other species in the genus *Colletotrichum* (data not shown). We then centered the search using the species most closely related to *C. graminicola* of graminicola and caudatum complexes **(Supplementary Table 1)**. Using the Hmmer v. 3.1 software suite (Finn et al., 2011), we located other proteins that had low similarity with CgEP3 (between 10.9% and 19.6%) but which were predicted to have NP domains, and some of them had an SP that overlapped with the NP domain (as in CgEP3) **(Supplementary Figure 2)**. However, because these proteins were larger (more than 300 amino acids in length) and they had low similarity with CgEP3, we conclude that this protein is specific to *C. graminicola.* In addition, it is possible that CgEP3 originated from ancestral gene duplication that occurred during the evolution of this fungus. This duplication could give two groups of proteins, including CgEP3 **(Supplementary Figure 2)**.

To better understand how the genomic region of *CgEP3* evolved, we constructed a species tree of *Colletotrichum* spp*.* **(Figure 22A)**. We selected 21 species of *Colletotrichum,* representing nine existing complexes **(Supplementary Table 1)**. *C. navitas* is the species most closely related to *C. graminicola* (Talhinhas & Baroncelli, 2021). We continued to investigate the synteny of the *CgEP3* genomic region, using genes that were upstream and downstream of *CgEP3,* as well as *CgEP3* itself*.* We used a TBLASTN search (Agarwala et al., 2016; Altschul et al., 1990; Boratyn et al., 2013) to identify these genes. In general, synteny was partially conserved in the graminicola and caudatum complexes **(Figure 22B)**. However, we found some genes that either were not present (without box) or were not conserved (white boxes) across many species. Compared with graminicola complex, synteny in other complexes were not conserved. Some examples include *GLRG_000875* and *GLRG_00876,* which were not found in all of the complexes (they were only found in graminicola and caudatum complexes, except *C. cereale*), and *GLRG_00877* and *GLRG_00878*, which underwent translocation in a cluster of species including *C. fructicola, C. truncatum, C. pluviorum,* and *C. orbiculare*. In the same way, when we analyzed the *CgEP3* region in greater depth, we did not find any conserved genes. Because there were no other proteins or genes that were similar to *CgEP3* in the other species we examined*,* we concluded that CgEP3 is specific to *C. graminicola.*

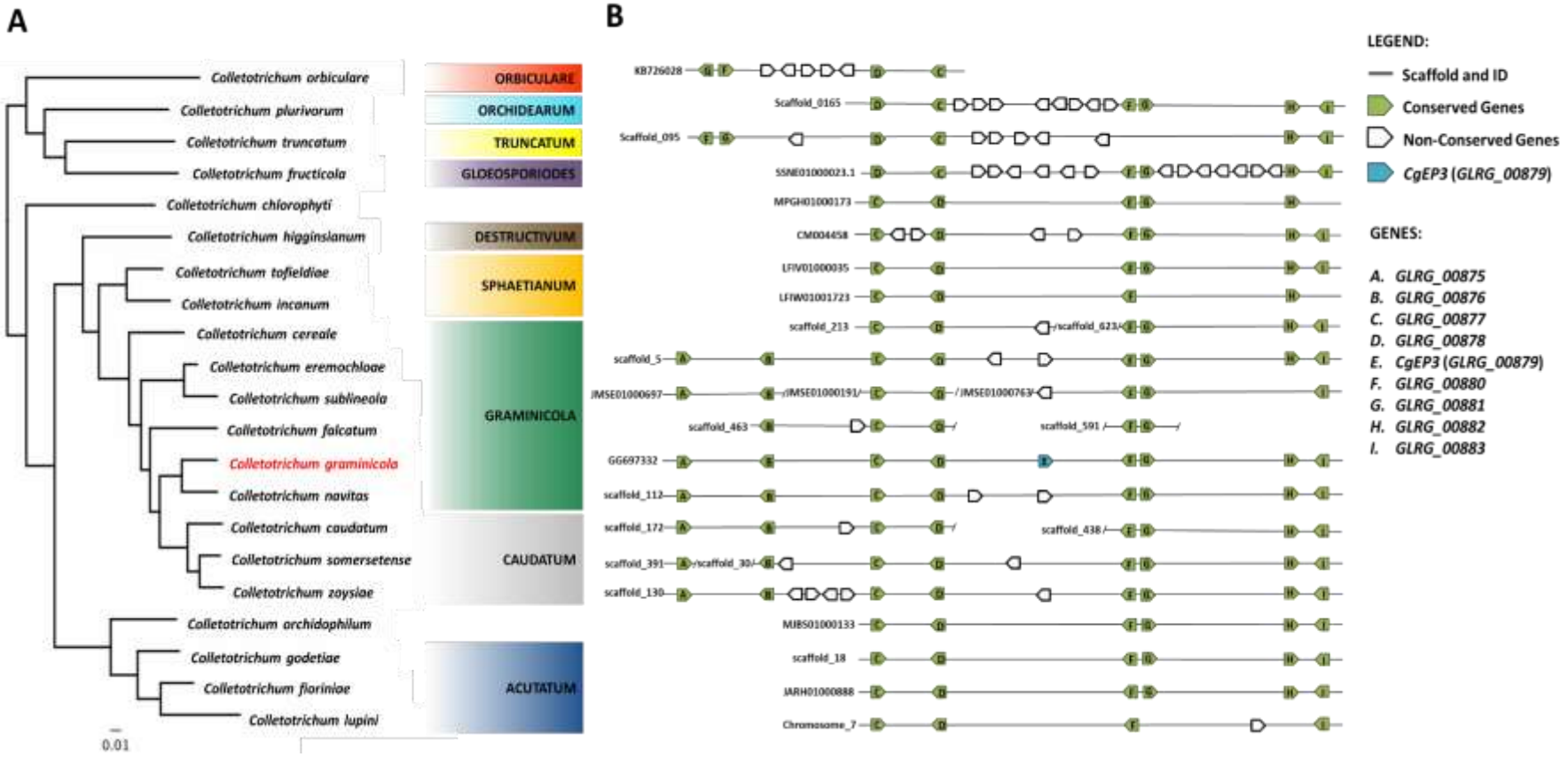
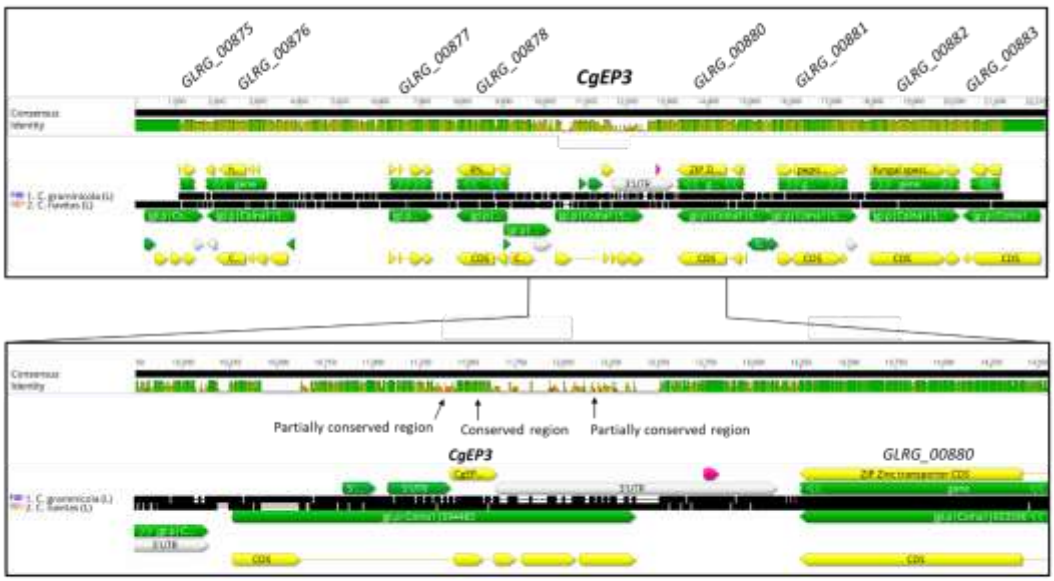


Figure 22. Synteny analysis of *CgEP3* genomic region. A) To better understand phylogenetic relationships among species of the genus *Colletotrichum*, a tree was constructed using the Markov Chain Monte Carlo algorithm and Bayesian inference. Multiple alignment was carried out using full sequences of the following genes: actin (*act*), chitin-synthase 1 (*chs-1),* and 3-phosphate dehydrogenase (*gadph*). *C. navitas* is the species most closely related to *C. graminicola* (Talhinhas & Baroncelli, 2021)**. B)** Synteny analysis aligned with a *Colletotrichum* species tree. Green boxes represent conserved genes, which have been designated with letters (A, B, C, D, F, G, H, I). *CgEP3* is framed in a blue box (E). White boxes without letters represent non-conserved genes. The scaffold of the genomic region is also identified. In general, synteny in the graminicola and acutatum complexes is partially conserved but was not conserved between complexes. We also did not find conserved genes comparable to *CgEP3* across *Colletotrichum* species. This fact suggests that CgEP3 is specific to *C. graminicola.*

CgEP3 may have evolved as a result of ectopic recombination

Following synteny analysis, we compared the genomic regions of *C. graminicola* and *C. navitas*. As previously mentioned, the upstream and downstream genes of *CgEP3* are conserved **(Figure 23A)**. With respect to *CgEP3,* a large part of the CDS is conserved as well as the 5' UTR and the final part of the 3' UTR. The final part of the 5' UTR, the initial part of the CDS, and the 3' UTR are partially conserved. In addition, when we compared the gene structures of *CgEP3* and *C. navitas jgi.p|Colna1|594485*, they presented several differences **(Figure 23B)**: in size (the *C. navitas* gene was bigger than the *C. graminicola* gene); in the number of exons and introns (*C. navitas* had five exons and four introns, whereas *C. graminicola* had one exon); in the presence of a predicted SP (*C. navitas* did not have a predicted SP but *C. graminicola* did); and in the characteristics of the NP domain (*C. navitas* had a typical NP domain, whereas *C. graminicola* had a partial NP-like domain). Because the gene structures are very different and the genomic region is partially conserved between both species, we concluded that *CgEP3* might be a result of ectopic recombination between different parts of the genome, especially because both regions were not homologous.

A



B

Specie	Genomic similarity	Nº Exons	Nº Introns	Protein Domains	Secreted Protein
C. graminicola	-	1	0	NP-Like	YES (SP predicted)
C. navitas	Partial	5	4	NP	NO

Figure 23. **Comparison of *CgEP3* genomic region in *C. navitas* and *C. graminicola.* A)** Above, MAFFT alignment (Katoh & Standley, 2013) of *CgEP3* genomic region of both species. The consensus identity bar represents the level of nucleotide sequence conservation: red, less than 30% identity; green-brown, at least 30% and less than 100% identity; green, 100% identity. Upstream and downstream genes in the *CgEP3* region are conserved. Below, the *CgEP3* region is indicated. Both regions are partially conserved. **B)** Principal differences of gene structures of *CgEP3* and *C. navitas jgi.p|Colna1|594485*. Taken together, these results suggest that the *CgEP3* region could have evolved quickly and that this could have been a result of ectopic recombination between different parts of the genome.

CgEP3 evolution in *C. graminicola*

To explore the evolution of *CgEP3* in *C. graminicola,* we selected 20 strains (unpublished data) (**Supplementary Table 2**). We performed a phylogenetic analysis of *CgEP3* and found that the strains differentiated into four clusters **(Figure 24)**. *C. graminicola* M1.001 was included in a cluster together with LARS 138, NRRL13649, and CBS 252.59. When compared to the rest of the strains, these four strains were found to have single nucleotide polymorphisms (SNPs) in the 5' UTR (including the intron), *CgEP3* CDS, and 3' UTR, resulting in a change from proline to threonine (pP7T). Thus, the *CgEP3* genomic region in this cluster could be subject to selective pressures and have evolved more quickly than that in the other clusters.

A study of the origin of the strains **(Figure 24 and Supplementary Table 2)** did not reveal any direct relationship between them. However, we could speculate about the USA strains. The blue cluster included the M1.001 and NRRL13649 strains, isolated from Missouri and Alabama, respectively. These two states are in the south of the USA. Another strain in the green cluster, NLRRL47511, was isolated from Michigan, which is in the north of the USA. It is likely that positive pressure could be higher in maize fields in the south of the USA than in the north; thus, the M1.001 and NRRL13649 strains could have evolved more quickly than the NRRL47511 strain in this genomic region. However, more *C. graminicola* strains from the USA are needed to confirm this hypothesis.

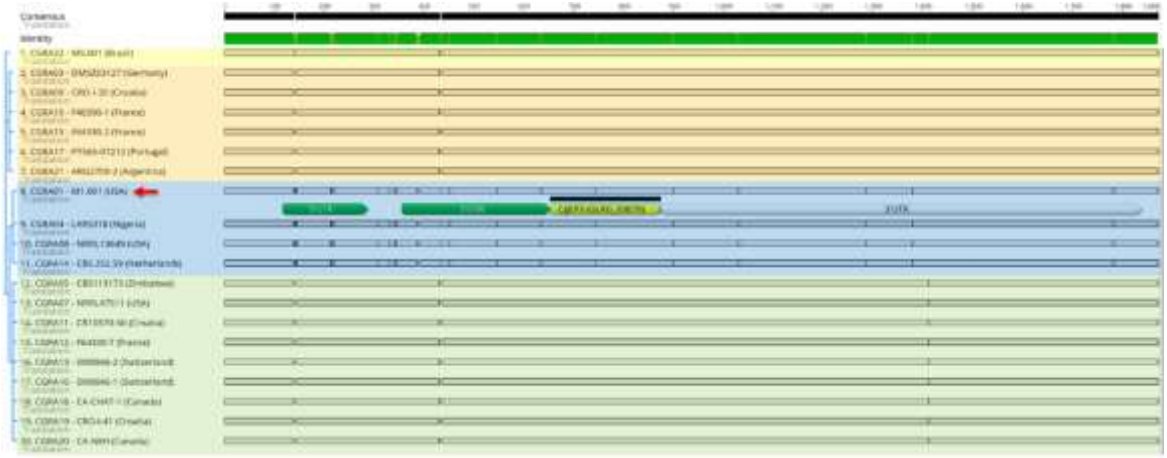


Figure 24. **Evolution of *CgEP3* in different *C. graminicola* strains.** A phylogenetic tree was constructed based on the *CgEP3* gene sequence of different strains of *C. graminicola* from around the world. There are four clusters of strains (yellow, orange, blue, and green). The M1.001 strain (CGRA01) is included together with LARS138 (CGRA04), NRRL13649 (CGRA08), and CBS 252.59 (CGRA14) strains in the blue cluster, which contain different SNPs in UTRs and CDS regions. The *CgEP3* genomic region in the blue cluster could be subject to selective pressures and has evolved more quickly than the rest of the clusters. The consensus identity bar represents the level of nucleotide sequence conservation: red, less than 30% identity; green-brown, at least 30% and less than 100% identity; green, 100% identity.

CgEP3 has a nucleo-cytoplasmic subcellular localization

To examine the localization of CgEP3 in maize host cells, we used two different approaches. First, we conducted an *in silico* analysis with the CgEP3 protein sequence using various online applications **(Supplementary Table 3)** and predicted that CgEP3 is a secreted protein with an SP of 22 amino acids (SignalP v5.0). To study the localization of CgEP3, we removed the fungal secretion signal following the methods described by Robin et al. (2018). The results showed that CgEP3 is an effector (EffectorP v1.0), without transmembrane domains (TMHMM Server v2.0), secreted (YLoc, BaCelLo, and Euk-mPloc v2.0), and may have nuclear localization (WoLFP SORT).

However, it did not have a predicted nuclear localization signal (Predict NLS, cNLS Mapper, and NLS Tradamus) or nucleolar localization signals (Nucleolus NoD).

The second approach was experimental determination based on the heterologous transient expression of CgEP3 in tobacco (*Nicotiana benthamiana*) plants infected with *Agrobacterium tumefaciens* (Robin et al., 2018) **(Figure 25)**. We carried out an N-terminal protein fusion of green fluorescent protein (GFP) with the CgEP3 sequence without its predicted SP (p*CaMV-35S::EGFP-CgEP3ΔSP*). This sequence is regulated by the 35S promoter of cauliflower mosaic virus (CaMV). Genes that are under the regulation of this promoter are constitutively expressed in plants (Amack & Antunes, 2020). Next, we transiently transformed tobacco plants through infection with *A. tumefaciens.* Results at 48 and 72 h post agroinfiltration showed that CgEP3 was localized in the nucleus and cytoplasm of tobacco plant cells **(Figure 25)**. However, additional experiments are needed to confirm this hypothesis. The protein fused with GFP can undergo cleavage processes and, as a consequence, the GFP diffuses to the nucleus. Thanks to the study of the integrity of the protein by western-blot using an anti-GFP antibody, the presence of the full length of the fused proteins can be detected. (Robin et al., 2018).

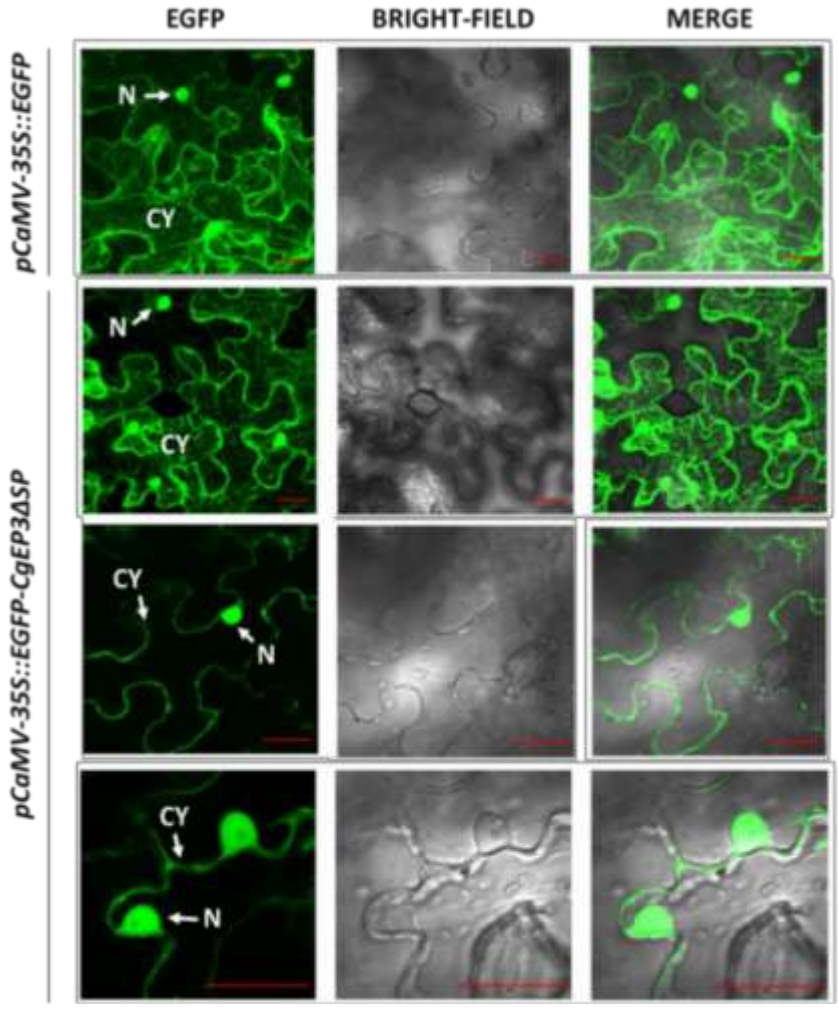


Figure 25. **CgEP3 has a nucleo-cytoplasmic subcellular localization.** *CgEP3* was transiently expressed through *A. tumefaciens* infection of *Nicotiana benthamiana* plants at 48 and 72 h post agroinfiltration. Confocal Z-stack projection and micrographs of CgEP3 (without SP) tagged with GFP (p*CaMV-35S::EGFP-CgEP3ΔSP*) showed that its localization was in the nucleus (N) and cytoplasm (CY). A GFP construct without any fused protein (p*CaMV-35S::EGFP*) was used as a positive control. Free GFP showed a nucleo-cytoplasmic distribution similar to that of CgEP3. Bars = 25 µM.

CgEP3 is expressed during early stages of infection and specifically in conidia, germ tubes, and appressoria

Infection of maize leaves with *C. graminicola* starts when a spore lands on the leaf surface and germinates. This process occurs approximately 12 h after the spores contact the plant. Next, the spore develops a germ tube at its tip, which forms appressoria throughout a period of 24 h. It has a melanized, dome-shaped structure. Subsequently, it forms a penetration peg that penetrates the plant epidermis. *C. graminicola* then begins to invade plant cells. The process takes around 24–36 h. When penetration is successful, bulbous primary hyphae start to colonize host cells, which is known as biotrophy (Gong et al., 2020; Gorman et al., 2020; Kleemann et al., 2008; Ludwig et al., 2014; Mims & Vaillancourt, 2002; R. J. O'Connell et al., 2012; D. Politis & Wheeler, 1972; Vargas et al., 2012, 2016). Primary hyphae with irregular shapes continue growing inside the plant cells and start to form branches that spread to other adjacent cells. At this stage of the disease, the fungus maintains live plant cells and does not produce symptoms (Gong et al., 2020; Micali et al., 2011; O'Connell et al., 2012; Panstruga, 2003; Perfect & Green, 2001; Sanz-Martín et al., 2016a). Finally, the fungus changes its behavior and begins to form thin secondary hyphae around 48–72 h. This is known as the necrotrophic phase. In this stage, *C. graminicola* kills the cells, and necrotic lesions start to form in infected tissues (Bergstrom & Nicholson, 1999; Gorman et al., 2020; Mims & Vaillancourt, 2002; R. J. O'Connell et al., 1985; Vargas et al., 2012; P. S. Wharton et al., 2001).

To determine the expression of *CgEP3* during maize leaf infection, quantitative RT-PCR (qRT-PCR) was performed at different time points (Sanz-Martín, 2016) **(Figure 26)**. Previous results showed that *CgEP3* was expressed in ungerminated conidia and during the early stages of infection, with a maximum at 12 and 24 hpi. During this time, conidia germinate to form germ tubes and appressoria in the biotrophic phase (**Figure 26)**. However, expression started to decrease quickly at 36 hpi. The expression values were very low compared to those at the beginning of the infection. *CgEP3* also showed low expression levels in saprophytically grown mycelia.

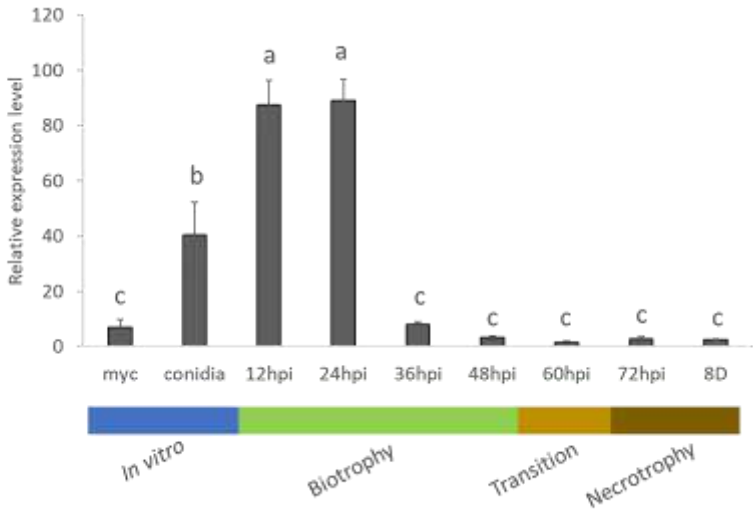


Figure 26. ***CgEP3* is expressed during early stages of infection.** To explore the expression level of *CgEP3,* we carried out a qRT-PCR analysis of leaves of maize infected with *C. graminicola* M1.001 at timelines of 12, 24, 36, 48, 60, and 72 hpi and 8 days post-inoculation. It also was checked in ungerminated conidia and in saprophytically grown mycelia (Sanz-Martín, 2016). These results obtained previously reported results that *CgEP3* is expressed in ungerminated conidia and during early stages of infection with a maximum at 12 and 24 hpi in the biotrophic phase. Afterwards, *CgEP3* expression decreased quickly at 36 hpi and continued to decline. It also had low expression in mycelia.

Normalization of expression values was carried out using *histone 3* and *α-actin* genes from *C. graminicola,* with 12 hpi samples used as a reference point for the analysis. Bars represent the average of measurements with ± standard deviation. The experiment was composed of two biological replicates, each with two technical replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD test. The bars with distinct letters represent statically significant differences ($P < 0.01$). This figure has been modified from the original that is found in the thesis dissertation of J. M. Sanz-Martín, 2016.

To improve the gene expression profile of *CgEP3* during the interaction of *C. graminicola* with maize, two transcriptional fusions of native promoters of *CgEP3* and *β-tubulin* with GFP were performed (p*CgEP3::GFP* and p*Cgβ-Tubulin::GFP*, respectively) (Sanz-Martín, 2016; Sanz-Martín, Pacheco-Arjona, et al., 2016). The p*Cgβ-tubulin::GFP* strain was used as a control. Using live imaging confocal microscopy, we observed the fluorescence of GFP and how *C. graminicola* developed during the infection of maize leaves **(Figure 27)**. At 12 hpi, the conidia started to germinate and develop germ tubes. We observed the GFP signal of p*Cgβ-tubulin::GFP* and p*CgEP3::GFP* strains in both structures. At 24 hpi, *C. graminicola* formed appressoria at the tip of the germ tube. Once again, GFP signals were detected in conidia, germ tubes, and appressoria. At 36 and 48 hpi, *C. graminicola* penetrated the plant epidermis and started to develop bulbous primary hyphae that spread to other adjacent cells. Unlike the p*CgEP3::GFP* strain, the p*Cgβ-tubulin::GFP* strain showed expression in primary hyphae at 36 and 48 hpi. Both strains still had a signal in conidia, germ tubes, and appressoria. Taken together, these results indicate that *CgEP3* is expressed during the early stages of maize leaf infection, specifically in conidia, germ tubes, and appressoria.

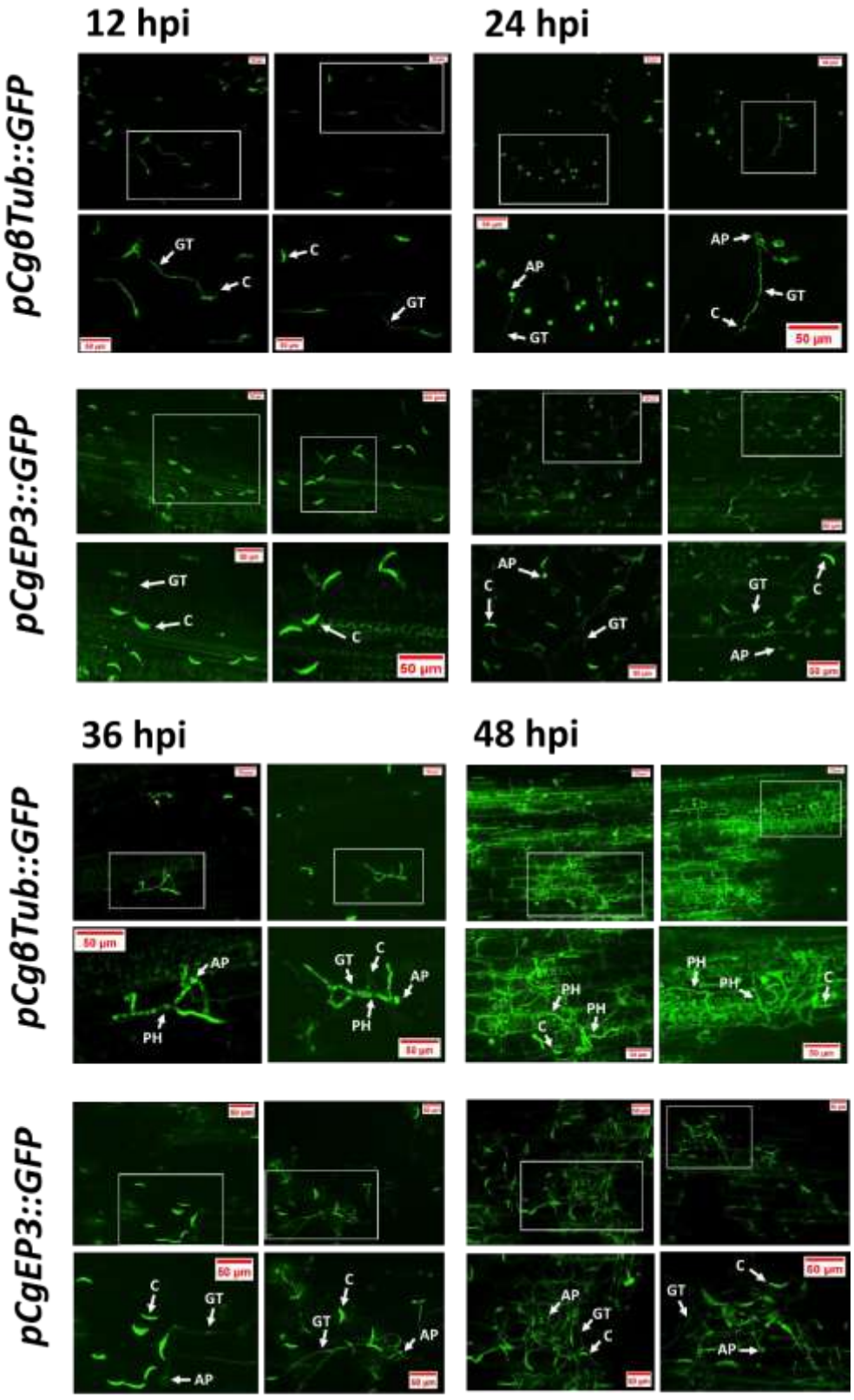


Figure 27. ***CgEP3* is expressed in conidia, germ tubes, and appressoria.** Live imaging micrographs obtained through confocal microscopy of maize leaves infected with *C. graminicola* p*Cgβ-Tubulin::GFP* and p*CgEP3::GFP* transcriptional fusion strains at 12, 24, 36, and 48 hpi. p*Cgβ-Tubulin::GFP* was used as a positive control because it is expressed constitutively and allows visualization of fungus development. GFP fluorescence signals were detected in conidia, germ tubes, and appressoria. This fact indicates that *CgEP3* expression is important during the early stages of infection. The white squares in the upper images designate the focus area of the lower images at the different time points. White arrows represent conidia (C), germ tube (GT), appressoria (AP), and primary hyphae (PH). Bars = 50 µM.

Generation of *CgEP3* transformant strains

To characterize the function of *CgEP3* during the development of maize anthracnose, we used different transformant strains. First, we generated a null mutant of *CgEP3* (Δ*CgEP3-18* strain) (Sanz-Martín, 2016) using the binary vector pKW1, a modified version of the vector utilized in the DelsGate methodology (García-Pedrajas et al., 2008; Vargas et al., 2016) **(Figure 28)**. Because of homologous recombination, the native *CgEP3* CDS was supplanted by a genetic construct with a hygromycin resistance gene (*hph*). It was also tagged with the *gfp* gene. Next, we complemented *CgEP3* with the pKW4 binary vector (Vargas et al., 2016). It has a nourseothricin resistance gene (*nat*). We generated two different constructs: 1) full complementation of the gene (Δ*CgEP3-18::CgEP3-8* strain) **(Figure 28)**, which included the *CgEP3* CDS and 1 kb upstream (promoter region) and downstream (termination region) genes of the *CgEP3* CDS; and 2) partial complementation of the gene (Δ*CgEP3-18::PC-CgEP3-49* strain) **(Figure 28)**. Because we did not know if the NP-like domain could fulfill its functions and because we thought that it was partial with respect to other NP domains, we partially complemented the sequence encoding the NP-like domain as well. Genetic construct included the native promoter region (1 kb upstream of *CgEP3* CDS), a partial CDS, and an artificial stop codon. Finally, we generated a strain that constitutively expressed the *CgEP3* gene (*Pgpda::CgEP3* strain) **(Figure 28)**. We utilized the USER™ Friendly cloning and pRF-HUE vector to obtain the construct (Frandsen et al., 2008). The *CgEP3* gene was under the regulation of the *gpda* of *Aspergillus nidulans.* These last three transformant strains were integrated into the genome through ectopic recombination. Transformant strains were analyzed using Southern blot analysis **(Supplementary Figure 3)** and PCR (data not shown).

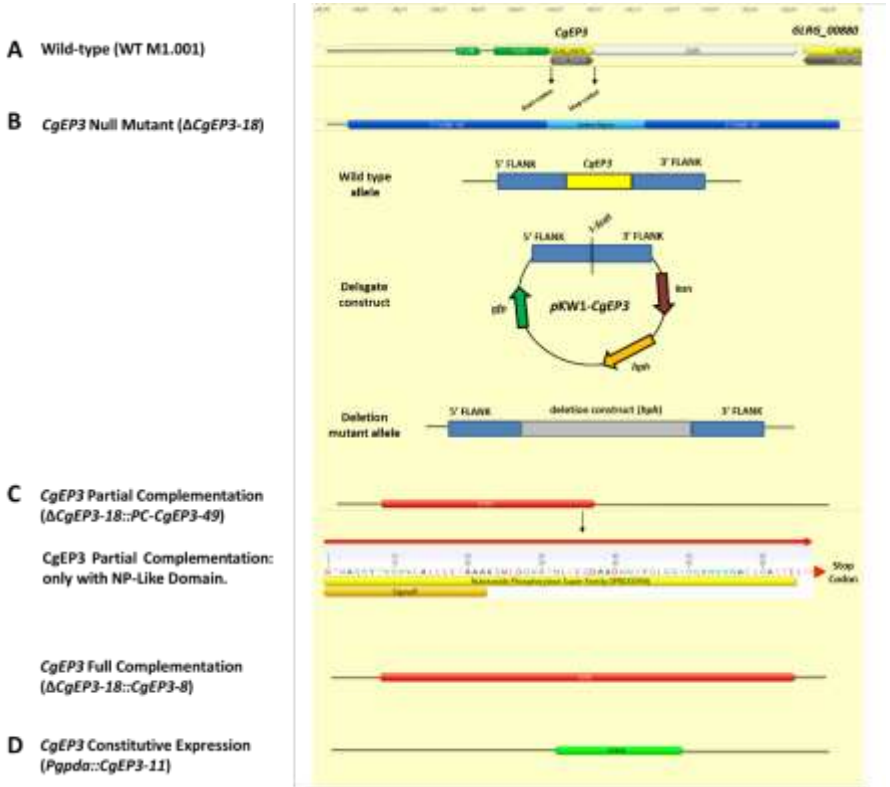


Figure 28. **Genetic constructs generated to characterize *CgEP3* gene. A)** *CgEP3* genomic region of *C. graminicola* M1.001 wild-type strain. **B)** Null mutant of *CgEP3* (Δ*CgEP3-18* strain) (Sanz-Martín, 2016). Above: The *CgEP3* genomic region that was deleted, along with its flanking 5' UTR and 3' UTR is shown. Below: a schematic of the Delsgate construct used for the deletion. It contains a I-*Sce*I restriction site and the hygromycin resistance gene. Through homologous recombination, native *CgEP3* was supplanted by the construct resulting in the mutant allele. **C)** We

carried out two types of complementation of *CgEP3*: full complementation (Δ*CgEP3-18::CgEP3-8* strain) and partial complementation (*ΔCgEP3-18::PC-CgEP3-49* strain), which included a partial *CgEP3* CDS with only the sequence encoding the NP-like domain (red arrow) with an artificial stop codon. We selected them using the nourseothricin resistance gene. **D)** Constitutive expression of *CgEP3* (*Pgpda::CgEP3* strain). *CgEP3* was under regulation of the *gpda* promotor of *A. nidulans.* We selected this strain using the hygromycin resistance gene. The last three transformant strains were integrated into the genome through ectopic recombination.

CgEP3 does not affect growth and development of *C. graminicola*

To characterize the transformant strains of *CgEP3* and to determine if they had any pleiotropic effects, we examined different growth and developmental traits. First, we studied conidia and appressoria morphology **(Figure 29A)**. We did not find any differences between the transformant and wild-type strains. Subsequently, we analyzed conidia and appressoria sizes **(Figure 29B)**. In both cases, the sizes of the transformant and wild-type strains were similar. We also checked the germination percentage of conidia growing *in vitro* and spore production. We did not observe any differences in the transformant and wild-type strains with respect to spore germination and production.

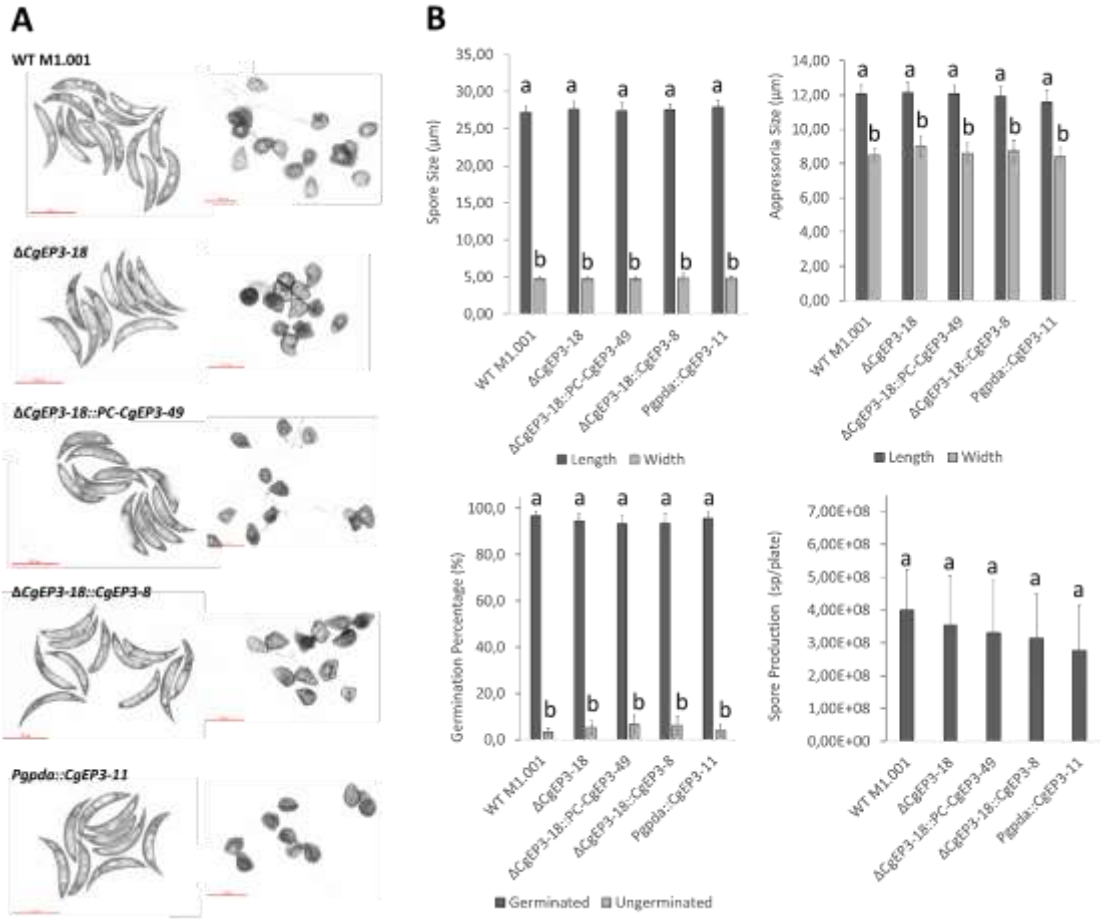


Figure 29. **Analysis of growth and development traits of conidia and appressoria of transformant strains. A)** Micrographs of conidia and appressoria were obtained using differential interference contrast microscopy. Bars = 20 µM (conidia), 10 µM (appressoria). They were previously stained with lactophenol blue solution. Transformant strains presented similar morphology for both structures. **B)** Above left and right, conidia and appressoria size. We did not find any differences in the analyzed transformant strains. Bars represent the average of measurements with ± standard deviation. Each experiment was composed of three biological replicates, each with three technical replicates. We assessed at least 50 conidia or appressoria per technical replicate resulting in 450 measures per strain.

Below left, conidia germination percentage. Values are similar for all transformant strains. Bars represent the average of measurements with ± standard deviation of an experiment composed of three biological replicates, each with three technical replicates. We assessed at least 100 conidia per technical replicate resulting in 900 measures per strain. Below right, spore production using 21 day-old PDA plates. We did not see differences among transformant strains. Bars represent the average of measurements with ± standard deviation. Each experiment composed of three biological replicates, each with four technical replicates. We assessed 12 counts per strain. For all cases, statistical analysis was performed using one-way ANOVA followed by Tukey's HSD test. The bars that have distinct letters represent statically significant differences ($P < 0.05$).

In addition, we tested mycelial growth *in vitro* in different media: potato dextrose agar (PDA), minimum agar (MA), and oatmeal agar (OMA) **(Figure 30)**. We did not find statistical differences in the average growth rate of transformant strains compared to the wild type.

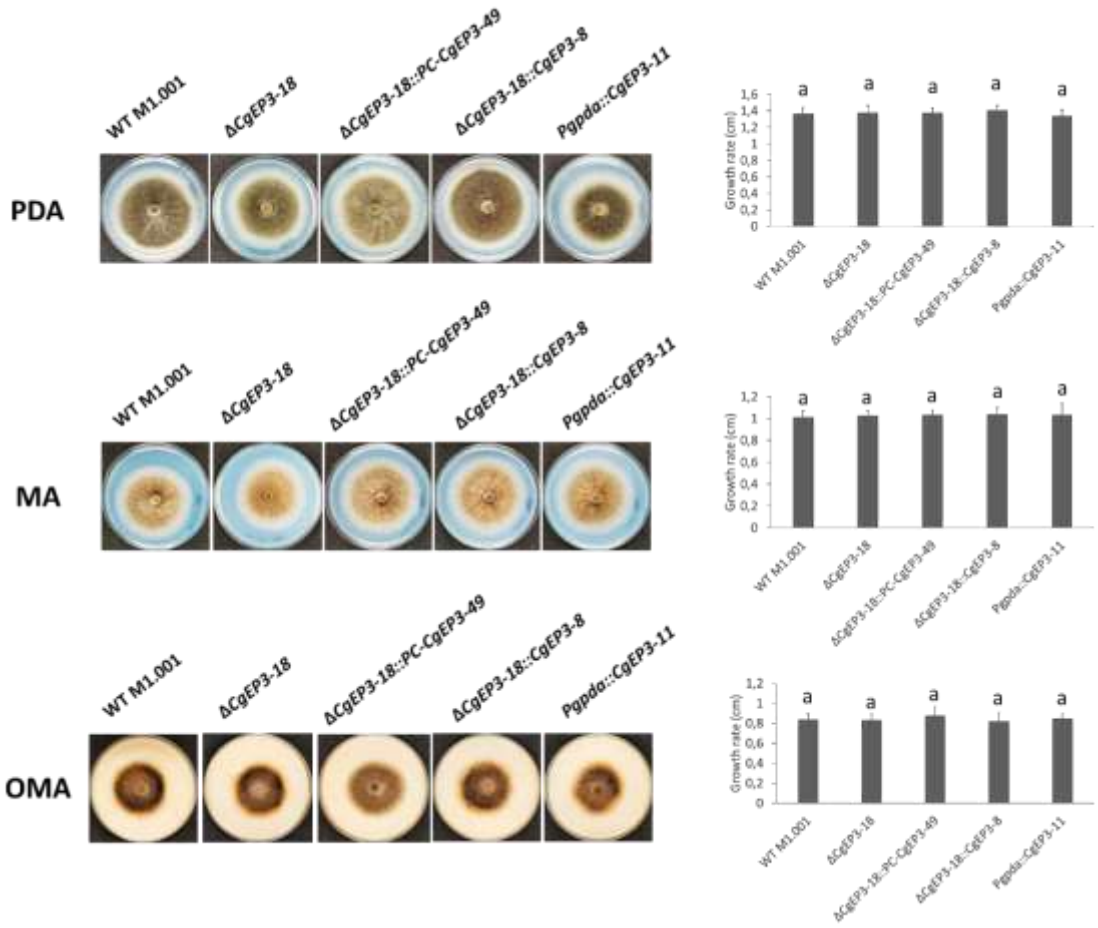


Figure 30. **Analysis of mycelial growth rate of transformant strains.** Mycelium was grown in different media: potato dextrose agar (PDA), minimum agar (MA), and oatmeal agar (OMA), during 6 days at 23˚C. We analyzed the growth rate. Bars represent the average of measurements with ± standard deviation. Each experiment was composed of three biological replicates, each with five technical replicates. We assessed 15 counts per strain. Statistical analysis was performed using non-parametric Mann-Whitney-Wilcoxon test. The letters of the bars are the same, indicating that there are no statistically significant differences among strains for the tested media ($P < 0.05$).

We concluded that *CgEP3* does not affect the growth and development of *C. graminicola,* and we did not observe pleiotropic effects in the different transformant strains (at least in the analyzed traits).

CgEP3 plays a role in *C. graminicola* virulence during the early stages of infection

We carried out infection of maize leaves and stalks following the protocols described by Vargas et al. (2012) and Eisermann et al. (2019). We inoculated a conidial suspension of different transformant strains on the surface of the third leaf of 3-week-old maize plants. We observed the symptoms of leaf blight at 3- and 4-days post-inoculation (dpi) **(Figure 31A)**. At 3 dpi, the lesion size in plants infected with the null mutant and partial complementation strains was smaller than that of plants infected with the wild-type. When we compared the lesion size of plants infected with the full complementation strain, it was similar to that of plants infected with the wild type. The initial phenotype was recovered, and *CgEP3* was implicated in the virulence of *C. graminicola* during the early stages of infection. Because the partial complementation strain did not recover the wild-type phenotype, restoring the full length of the protein is probably necessary for it to re-establish its function. Similarly, we did not observe any difference in lesion size in plants infected with the constitutive expression strain compared with that in the wild type-infected plants. Constant gene expression did not produce changes in phenotype. At 4 dpi, lesion size was similar in plants infected with transformant strains compared with that in the wild type-infected plants. This could be because the fungus could release other effectors and virulence factors that are able to supplement the function of CgEP3. We also pipetted a conidia suspension inside a cavity that was made at the first internode of 6-week-old maize plants **(Figure 31B)** and observed stalk-rot symptoms. The results were correlated with those obtained previously for leaf blight infection at 3 dpi. We concluded that *CgEP3* plays a role in virulence and is important during the early stages of maize infection.

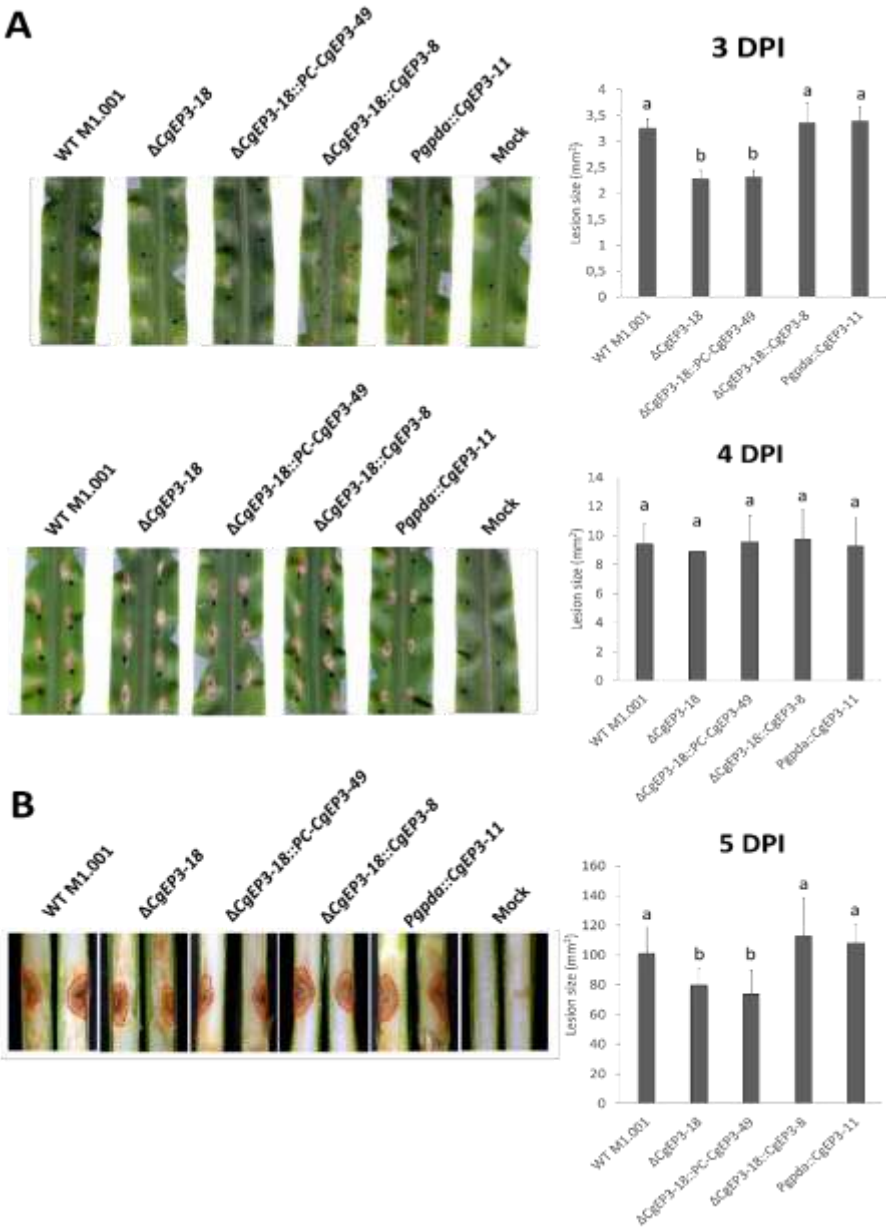


Figure 31. **Anthracnose leaf blight and stalk-rot symptoms in maize plants infected with *CgEP3* transformant strains:** **A)** The third leaf of each maize plant was infected with spore suspension of 3 x 10^5 sp/mL. Results were assessed at 3 and 4 dpi. The black dots indicate the inoculation points. Bars represent the average of measurements with ± standard deviation. Each experiment was composed of three biological replicates, each with four technical replicates. We assessed 20 infection spots per replicate resulting in 320 measures per strain. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD test. The bars that had distinct letters represent statically significant differences ($P < 0.05$). **B)** Maize stem infection with the inoculation of a spore suspension of 2 x 10^6 sp/mL. Results were analyzed at 5 dpi. Bars represent the average of measurements with ± standard deviation of an experiment composed of three biological replicates, each with five technical replicates. We assessed at least 15 plants per replicate. Statistical analysis was performed using non-parametric Mann-Whitney-Wilcoxon test. The bars that have distinct letters represent statistically significant differences ($P < 0.05$). For both experiments, Paint.NET v4.2.16 (dotPDN, LCC) was used to measure the lesion size.

C. graminicola leaf penetration and biomass accumulation are reduced during infection

Because we observed that *CgEP3* played a role in fungal virulence, we decided to study the behavior of the null mutant during infection. Thus, we analyzed fungal colonization. Previously, a quantitative assay of fungal biomass was performed using qRT-PCR (Weihmann et al., 2016).

The results showed that fungal biomass was reduced in plants inoculated with the null mutant compared to that in the wild type-infected plants at 3 dpi **(Figure 32A)** (Sanz-Martín, 2016). We analyze the level of penetration during leaf infection at 36 hpi **(Figure 32B)**. The level of penetration by null mutant strain decreased by 19% compared to that of the wild-type strain. We concluded that CgEP3 could help the fungus during penetration of the plant epidermis and colonization of plant leaf tissue and allow the disease to advance, while its lack would produce a delay in disease development in the null mutant-infected plants.

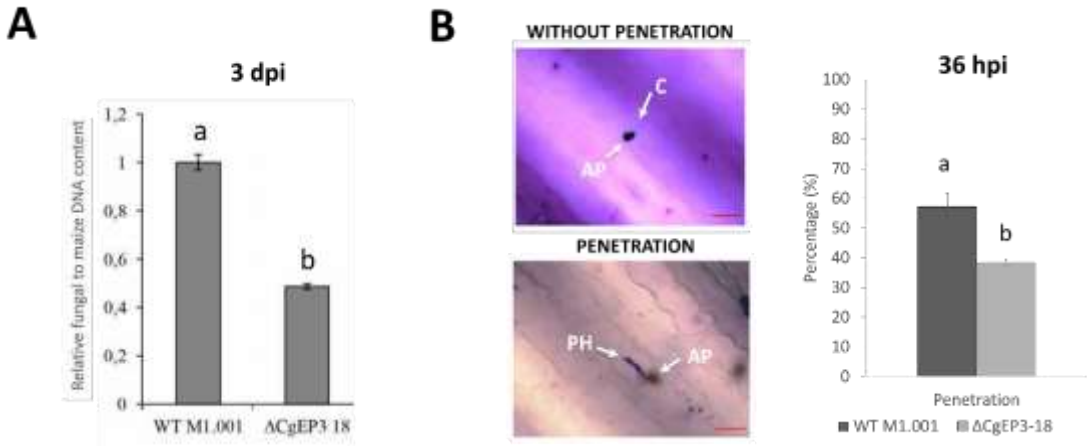


Figure 32. **Biomass and penetration level of the null mutant strain were reduced during infection of maize leaves.** **A)** Fungal biomass of plants inoculated with the null mutant and wild-type strains at 3 dpi. It was assessed previously using qRT-PCR (Sanz-Martín, 2016). Bars represent the average of measurements with ± standard deviation. Each experiment was composed of two biological replicates, each with three technical replicates. Statistical analysis was performed using an unpaired *t* test. The bars that have distinct letters represent statistically significant differences ($P < 0.01$). This figure has been modified from the original that is found in the thesis dissertation of Sanz-Martín, 2016. **B)** Penetration percentage level of maize leaves infected with the null mutant and wild-type strains at 36 hpi. Micrographs were obtained using light microscopy of leaf tissues stained with toluidine blue and aniline blue. This is an example of an appressorium of the *C. graminicola* M1.001 wild-type strain that could not penetrate leaf epidermis (above) and another that does penetrate and starts to develop primary hypha (below). White arrows represent conidia (C), appressorium (AP), and primary hypha (PH). Bars = 25 µM. Bars represent the average of measurements with ± standard deviation of the experiment composed of three biological replicates. We assessed at least 100 appressoria per replicate resulting in 300 measures per strain. Statistical analysis was performed using an unpaired *t* test. The bars that have distinct letters represent statically significant differences ($P < 0.05$).

CgEP3 does not affect papillae apposition during the infection of maize leaves

We also explored papillae appositions during maize leaf infection at 36 hpi **(Figure 33)**. Papillae appositions that have a dome shape are deposited by the plant epidermal cells into the apoplasm (space that is found between the plasma membrane and the cell wall). The fungal appressoria and penetration peg are directly subtended by papillae, and the penetration peg can often become trapped in the matrix formed by the papillae (Assaad et al., 2004; Ebrahim-Nesbat et al., 1986; Hippe-Sanwald et al., 1992; Kunoh et al., 1996). This matrix is impregnated with different compounds such as hydrolases, reactive oxygen species, and phenolic compounds that accumulate in the papillae. This leads to the creation of an effective barrier against the penetration of fungi (Assaad et al., 2004; Bélanger et al., 2002). We divided appressoria into different categories depending on whether they could penetrate leaf epidermis and whether the plant deposited papillae appositions (categories I-IV). We did not find any differences between plants infected with the null mutant strain and the wild-type strain with respect to

papilla apposition (category III). In most of the appressoria that achieved successful penetration, maize plants deposited papillae appositions surrounding the primary hyphae (category IV). Thus, we believe that CgEP3 does not affect papilla apposition in the plant defense response.

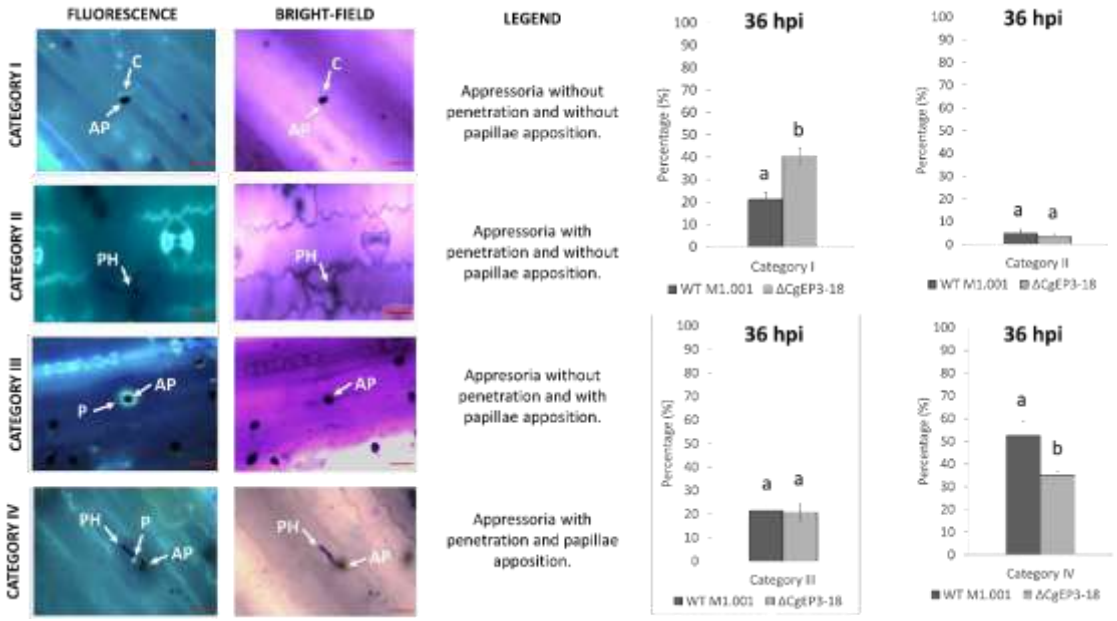


Figure 33. **Papillae apposition during the appressoria penetration in maize infected leaves at 36 hpi.** We assigned appressoria in different categories (I-IV) depending on penetration and papillae appositions (legend). Micrographs were obtained using epifluorescence and light microscopy of leaf tissue stained with toluidine blue and aniline blue. White arrows represent conidia (C), appressorium (AP), primary hypha (PH), and papillae appositions (P). Bars = 25 µM. Bars represent the average of measurements with ± standard deviation. Each experiment was composed of three biological replicates. We assessed at least 100 appressoria per technical replicate, resulting in 300 measures per strain. Statistical analysis was performed using an unpaired *t* test. The bars that have distinct letters represent statically significant differences ($P < 0.05$).

Appressorial internal turgor pressure is enough to penetrate plant epidermis

Because we discovered that the null mutant strain penetrated less than the wild-type strain, we decided to study appressorial cell wall rigidity. We analyzed the phenomenon of cytorrhysis **(Figure 34)**. When a cell (in our case, appressorium) is subjected to a high molecular weight compound, it cannot cross the wall and consequently produces compressive stress on the cell. Then the cell becomes dewatered and collapses. This phenomenon is known as cytorrhysis (Money et al., 1998).

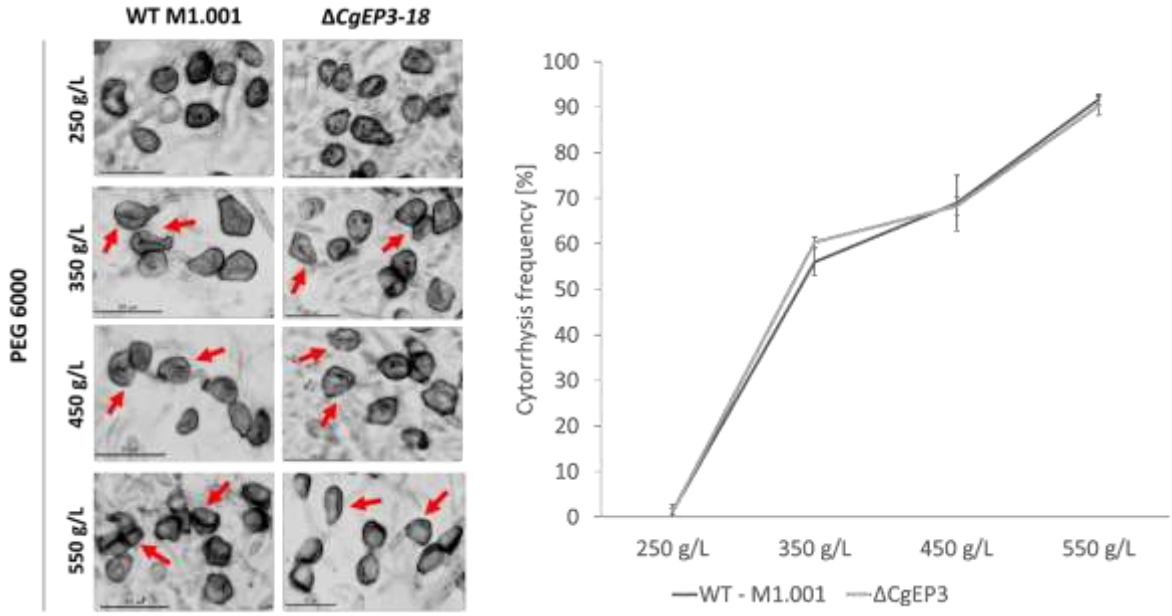


Figure 34. **Study of appressorial cell wall rigidity through the phenomenon of cytorrhysis.** We exposed appressoria of null mutant and wild-type strains growing *in vitro* to different concentrations of a high-molecular-weight compound, polyethylene glycol 6000 (PEG 6000). Micrographs were obtained using a light microscope. Red arrows represent the collapse of appressoria cell walls (cytorrhysis). Bars = 20 µM. Data represent the average of measurements with ± standard deviation. Each experiment was composed of three biological replicates. We assessed at least 100 appressoria per technical replicate, resulting in 300 measures per strain.

The results showed that when the concentration of polyethylene glycol 6000 (P6000) was low, at 250 g/L, cytorrhysis was not produced in either the null mutant or wild-type strains. However, at the increased P6000 concentration, the appressoria cell wall started to collapse. When we observed the behavior of the cytorrhysis frequency curve, there were no differences between the null mutant and wild-type strains. We concluded that appressorial cell wall rigidity in both strains was similar, and that internal turgor pressure was sufficient to penetrate the plant epidermis.

In addition, we stained appressoria of the null mutant and wild-type strains growing *in vitro* with Nile red **(Figure 35)**, which can dye lipid droplets inside appressoria. It can also bind to neutral triglycerides, which makes it useful in the study of lipid dynamics (Eisermann et al., 2019; Greenspan & Fowler, 1985; Z. Wang et al., 2018). Bhadauria et al. (2012) discovered that the ATG1 protein is important in lipid mobilization and contributes to pathogenicity in *Magnaporthe oryzae.* To generate turgor pressure during the penetration of appressoria, it is necessary to translocate and degrade lipids (formed by droplets or bodies) inside appressoria. This is known as lipolysis and occurs inside the vacuoles of maturing appressoria. As a result of lipid degradation, fatty acids and glycerol (a highly soluble osmolyte) are produced. These compounds are required to mechanically breach the plant surface (Bhadauria et al., 2012). Therefore, we analyzed the appressorial lipid dynamics in the null mutant strain compared to that of the wild-type strain **(Figure 35)**. We observed a population of heterogeneous lipid vesicles inside the appressoria. In general, we did not find large differences between both strains in, for example, the number of vesicles, their size, or position. Therefore, we conclude that lipid mobilization into appressoria was optimal, and lipid reserves would be sufficient for appressorial penetration.

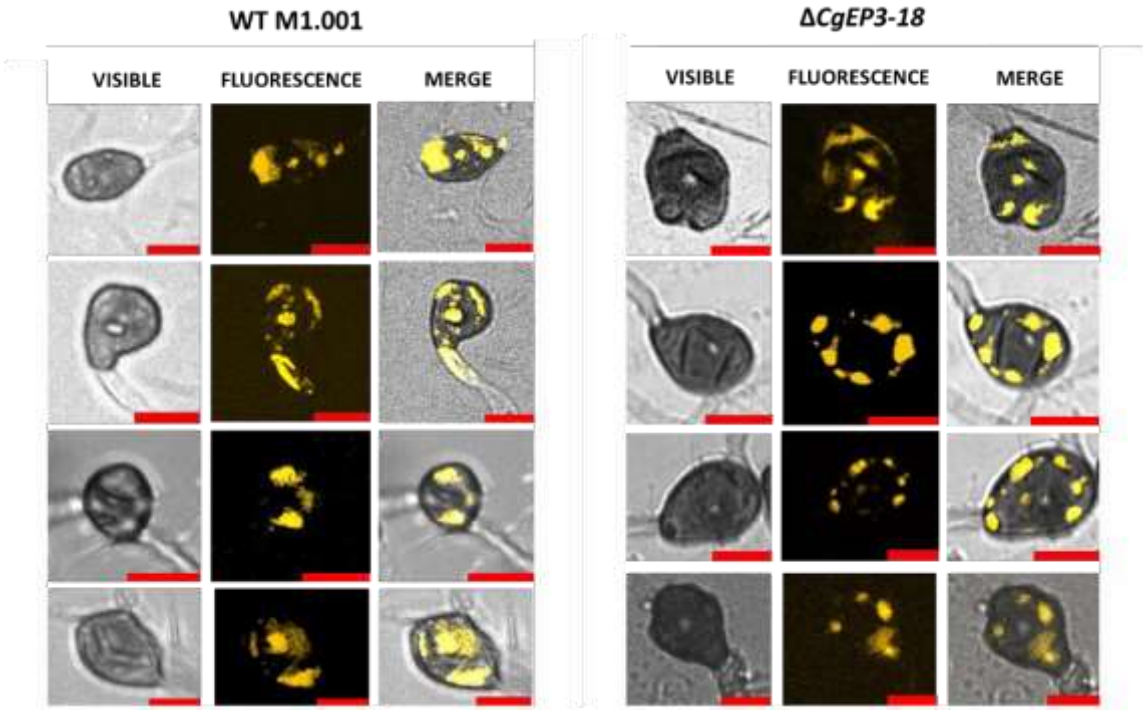


Figure 35. **Analysis of lipid dynamics inside appressoria.** We grew appressoria of null mutant and wild-type strains *in vitro* and then stained them with Nile red. Micrographs were using through epifluorescence microscopy. Bars = 10 µM. The experiment was composed of three biological replicates. We assessed at least 50 appressoria per technical replicate, resulting in 150 measures per strain.

DISCUSSION

Pathogens can release effectors to avoid or suppress plant defense responses through the manipulation of plant immunity, physiology, and pathogen surveillance systems. Effector proteins are genetically diverse because in the evolution of host-pathogen interactions there has been an arms race that has made their adaptation possible (S. Kim et al., 2020). Depending on where they function in the cell, proteinaceous effectors can act in the cytosol or translocate to other compartments of the plant cell, such as the nucleus. Here, effectors can link to DNA and utilize the transcriptional machinery of the host to disturb the expression of genes and, consequently, their immune response (Figueroa et al., 2021). To date, few fungal nuclear effectors have been characterized. Among the *C. graminicola* nuclear effectors, CgEP1 is the only one that has been characterized. It binds to host DNA and plays an important role during the biotrophic stage. It is necessary for the development anthracnose disease in the leaves, stalks, and roots (Vargas et al., 2016). Other nuclear effectors have been described using the agroinfiltration method in *N. benthamiana*: CgCFEM6 and CgCFEM8, which partially or completely suppress cell death, respectively (Gong et al., 2020), and CgCEC3, which can induce a weak defense response and nuclear expansion (Tsushima et al., 2021). In this study, we characterized the *C. graminicola* CgEP3 effector candidate, which impacts virulence during the early stages of maize infection. Based on *in silico* and experimental analyses, we showed that CgEP3 is a secreted protein that has nucleocytoplasmic subcellular localization.

CgEP3 contains a predicted partial NP-like domain. Nucleoside phosphorylase proteins play a role in nucleoside phosphorolysis, which is reversible, as well as in transferase reactions involving pyrimidine or purine bases (Carballeira et al., 2009). When N-glycosidic bonds are broken in different molecules such as the corresponding nucleoside and sugar-1-phosphate, they can be utilized as energy and carbon sources, as well as for the synthesis of nucleotides (Gojic, 2018; Takehara et al., 1995). NPs could be important for virulence in different organisms because of their role in nucleotide recycling and salvage pathways, for example, in the fungus *Blastomyces dermatitis* and *B. gilchristii* (causing blastomycosis in humans) (Muñoz et al., 2015), intracellular parasite microsporidian *Encephalitozoon romaleae* (Selman et al., 2011), and protozoan parasite *Plasmodoium yoelii* (a model organism for studying human malaria) (Ting et al., 2008). NPs are also regulators of defense against intracellular and extracellular pathogen infections in the nematode *Caenorhabditis elegans* (Tecle et al., 2021). Nevertheless, the function of NP proteins in plant-pathogen interactions remains unclear. The CgEP3 NP-like domain has a smaller size than that of the NP domains usually found in the genus *Colletotrichum*. Thus, the NP-like domain might be partial and probably does not have any function or else retains only some of the NP functions. This hypothesis is supported by the results we obtained during the infection of maize leaves and stalks. The partially complemented strain of the gene (only with NP-like domain) did not recover the virulence phenotype compared to the plants infected with the wild line. However, further investigations are required to determine if this domain fulfills its function.

Effectors are important for host specialization (F. E. Hartmann et al., 2017; L. J. Ma et al., 2010; Poppe et al., 2015; Raffaele et al., 2010; Sánchez-Vallet et al., 2018). Pathogens evolve to avoid recognition by the host, and this is achieved through functional optimization, such as alteration of sequences, deletion of genes, modification of effectors, and evolution of new effectors. Thus, the pathogen can remain virulent and continue to infect its host (Lo Presti et al., 2015; Sánchez-Vallet et al., 2018). CgEP3 is a novel effector specific to *C. graminicola*. Its protein sequences were not similar to the sequences of the NP proteins of the graminicola species complex.

However, we found other proteins that had an SP that overlapped with an NP domain and shared a similar structure with CgEP3. Furthermore, CgEP3 may have evolved from an ancestral gene duplication. Following duplication, both copies continue evolving, and one of them can diverge until its homology is not detectable, as with CgEP3. At the same time, the gene and its associated protein(s) can evolve to perform different functions through a process known as sub-/neofunctionalization (Fouché et al., 2018; Freeling et al., 2015; Plissonneau et al., 2017; S. Tanaka et al., 2019). Thus, owing to strong selection pressure during evolution, CgEP3 may have evolved from an ancestral nucleoside phosphorylase. Then *CgEP3* may diverged to encode an effector protein. There are some examples of *C. graminicola* effectors that have evolved after gene duplication, such as CgEP1 and its paralog GLRG_09337 (Vargas et al., 2016). In addition, there are CgCFEM effectors, whose expansion of CFEM domains through fungal genomes depended on both domain and gene duplications during recombination (Gong et al., 2020). Subsequent synteny analysis of the *CgEP3* genomic region confirmed the hypothesis that *CgEP3* is specific to *C. graminicola.* The *CgEP3* nucleotide sequence did not have broad similarities with related genomic regions of the graminicola complex. Compared with the genomic region of the most closely related species, *C. navitas*, the *CgEP3* region evolved quickly, and this was a result of ectopic recombination between different parts of the genome. The localization of effectors in the genome of pathogens is frequent in repeat-rich genomic compartments (S. Dong et al., 2015b; Fouché et al., 2018), which have a significant number of polymorphisms. These polymorphisms can cause effectors to evolve more rapidly by augmenting the non-homologous recombination frequency, and consequently, the appearance of duplications and deletions in the effector genes. Thus, the variations found in these chromosomal regions promote the evolution of effectors. However, there are exceptions, and some effector genes are not found in chromosomal regions that evolve quickly (Fouché et al., 2018), similar to *CgEP3.* This fact has been observed in different studies of the *Magnaporthe oryzae, Fusarium graminearum,* and *Leptosphaeria maculans* genomes (Brown et al., 2012; Dean et al., 2005; Fouché et al., 2018; Gervais et al., 2017). Additionally, evolutionary analysis of *CgEP3* in several *C. graminicola* strains showed a cluster of four strains (M1.001, LARS 138, NRRL13649, and CBS 252.59), in which *CgEP3* is subject to selective pressure, and it has evolved more quickly than that of the rest of the strains. This fact is represented by the accumulation of SNPs in the 5' UTR (including the intron), CDS, and 3' UTR of *CgEP3.* Additionally, we can speculate about the group of strains isolated from the USA. Selective pressure may be higher in maize fields in the south of the USA than in the north; thus, the M1.001 and NRRL13649 strains may have evolved more quickly than the NRRL47511 strain in this genomic region. However, further study of more *C. graminicola* strains from the USA is needed to confirm this hypothesis. This suggests local adaptation (Richards et al., 2019) and positive selection may have helped *C. graminicola* adapt to local conditions in the USA.

Many effectors have expression profiles that present a characteristic peak during host infection, either when the pathogen is established or during growth inside the host (Hacquard et al., 2013; R. J. O'Connell et al., 2012; Palma-Guerrero et al., 2016; Skibbe et al., 2010). *CgEP3* is expressed during the early stages of infection, and at a maximum between 12 and 24 hpi in the biotrophic phase, and specifically in conidia, germ tubes, and appressoria. Other effectors are also expressed early in plant-pathogenic fungal interactions (Irieda et al., 2014; Kleemann et al., 2008; Shang et al., 2020; M. F. Torres et al., 2016). During this period, *C. graminicola* can express numerous genes, particularly during the maturation of appressoria, host penetration, and colonization. Before penetration, *C. graminicola* starts to participate in an "intimate and dynamic conversation" with the maize cells (M. F. Torres et al., 2016). This implies that the

communication is not one-way, and the signals that both plants and fungi receive are important. The plant produces signals that can trigger the development of the fungus, and vice versa. The fungus can generate signals that lead to responses in the maize (M. F. Torres et al., 2016). Thus, CgEP3 could be important in host-pathogen interactions during the early biotrophic phase.

In this study, we demonstrated that CgEP3 plays a role in the virulence of *C. graminicola* during maize leaf and stalk infection. The deletion of *CgEP3* contributes to a decrease in lesion size in leaves and stalks during the early stages of infection. We also observed that maize plants infected with a partial complementation strain did not recover the wild-type phenotype. Thus, *C. graminicola* needs the full-length protein to restore its function. A similar case was reported by Tanaka et al. (2014). They studied the *Ustilago maydis* Tin2 effector, which participates in the production of anthocyanin during the infection of maize. In maize plants infected with the fungus strain lacking *Tin2*, anthocyanin was induced, and vascular tissue was not colonized by the pathogen. They complemented the mutant with different versions of the protein: $Tin2_{1\text{-}207}$, with the full sequence of the protein, and $Tin2_{1\text{-}206}$, a truncated version missing only the last amino acid. When plants were infected with both complemented strains, the strains recovered their virulence phenotypes. However, when two other truncated versions, $Tin2_{1\text{-}202}$, without five amino acids in the C-terminus region, and $Tin2_{AAAAA}$, in which the last five amino acids of the sequence were replaced with alanine, were used as complements, neither complemented strain regained its virulence phenotype. Hence, the C-terminus region of Tin2 is necessary during the induction of anthocyanin production and the formation of tumors. Our case is similar; thus, CgEP3 could require an additional seven amino acids (RDRRGIS) in the C-terminal region to restore the wild-type phenotype. Moreover, constitutive expression of CgEP3 did not affect virulence. This effect was observed in two previously studied effectors in the group, CgEP1 and Cgfl (Sanz-Martín et al., 2016a; Vargas et al., 2016). The level of proteins, as well as their enzymatic activity, might not correlate with the level of transcripts. There are several processes that regulate protein translation, post-translational changes, and protein replacement (Baeza-Montañez et al., 2015; Sanz-Martín et al., 2016a; Yang et al., 2013). Although the cell constantly expresses the gene, it is likely that either the protein is not translated or the protein is not functional (Sanz-Martín, Pacheco-Arjona, et al., 2016). We also analyzed the growth and development traits of the *CgEP3* transformant strains. We did not observe changes in phenotype in the different strains, and they did not have any pleiotropic effects (at least for the studied traits). Thus, CgEP3 does not participate in the growth and development of *C. graminicola,* but it could be important during plant-pathogen interactions.

Once we confirmed that CgEP3 contributed to virulence, we continued to analyze null mutant behavior during infection. We discovered that it accumulated less fungal biomass than the wild type during the colonization of maize leaves. In addition, the null mutant had a reduced level of penetration compared to that of the wild type during maize leaf infection, which delayed disease development (as we saw during leaf blight and stalk rot assays). Thus, we hypothesized that CgEP3 could help *C. graminicola* during plant epidermis penetration. To study the penetration process in appressoria in greater depth, cytorrhysis and lipid dynamic experiments revealed that internal turgor pressure was sufficient to penetrate the plant epidermis in the null mutant strain. CgEP3 did not play a role in apical cell wall rigidity and lipid mobility. Based on these results, we concluded that CgEP3 acts as a pre-penetration effector. In *C. higginsianum,* the expression of numerous ChEC effectors was upregulated during pre-invasion of its host *Arabidopsis thaliana.* Before plant colonization, effectors that are expressed early during infection are released from the penetration pores of appressoria (Kleemann et al., 2012). They studied two effectors, ChELP1 and ChELP2, both of which have chitin-binding LysM domains. They are necessary for

the penetration of appressoria and to abolish chitin-triggered responses (Robin et al., 2018; Takahara et al., 2016). Furthermore, the MC69 effector of *C. orbiculare* (a pathogen of cucumber) is required for successful fungal infection. The *Δmc69* mutant strain showed reduced penetration (Irieda et al., 2014; Saitoh et al., 2012). Additionally, the orthologous effector protein Clu5a in *C. graminicola* is required to penetrate the epidermal cell wall (Eisermann et al., 2019). Both the Clu5a and Clu5d effectors are specifically required in pathogenesis, and both are expressed prior to plant invasion (Eisermann et al., 2019). In contrast, *C. fructicola CgEC92* is expressed in appressoria during the early stages of apple leaf infection. CfEC92 can help the fungus during the penetration of appressoria and enhances virulence by suppressing the PTI response in plants (Shang et al., 2020). Finally, ClCE8, an effector of *C. lentis,* (a pathogen of lentil) was induced specifically during appressorium penetration. It may mediate downstream infections related to fungal development (Bhadauria et al., 2015). These pre-penetration effectors of different *Colletotrichum* species could help fungi penetrate their host and modulate early responses during infection. Effectors that are expressed early can help the fungus to avoid plant defense before invasion and assist with the colonization of plant cells (Kleemann et al., 2012). Additionally, these effectors can play a role as "anesthetic agents" lulling the plant cells into relaxing their defense. Then, the penetration process can be completed with minimal resistance (Dou & Zhou, 2012). CgEP3 plays a similar role and contributes to the suppression of early plant defense. Transcriptional profiling experiments were performed and are described in Chapter II of this thesis. Additionally, we observed that CgEP3 had no effect on papilla apposition during plant penetration. As in other cases, if mature papillae were deployed, the mutant strain could break them. This fact enables the fungus to have increased access to plants (Eisermann et al., 2019; Mims & Vaillancourt, 2002; Weihmann et al., 2016).

In summary, a comprehensive analysis of the *C. graminicola* proteome (Vargas et al., 2016) led to the discovery of a novel pre-penetration effector localized to plant's nucleus and cytoplasm. To the best of our knowledge, CgEP3 is the first species-specific effector to be described in *C. graminicola.* It may have evolved from an ancestral gene duplication event and diverged to encode and effector proteins whose ancient function was to serve as a nucleoside phosphorylase. Furthermore, synteny analysis revealed that the *CgEP3* genomic region may have evolved quickly, which could be a result of ectopic recombination between different parts of the genome. We propose that CgEP3 plays a role in virulence during the early stages of infection of *C. graminicola* and helps the fungus penetrate the host epidermis. CgEP3 could avoid maize-preinvasion defense and help the fungus colonize plant cells with very little resistance. Because it causes anthracnose disease, *C. graminicola* represents an incipient threat to agriculture and is especially important as climate change and maize demand are both increasing. A better understanding of host-pathogen interactions is vital for implementing appropriate crop management strategies against this disease (Balmer et al., 2013). Thus, an understanding of how effectors act in the host could be used in crop improvement programs as well as in the selection of anthracnose-resistant germplasm to enhance crop health (Dangl et al., 2013; Fleur Gawehns et al., 2013; Pavan et al., 2010; Sanz-Martín, 2016; Vleeshouwers & Oliver, 2014). Because CgEP3 is exclusive to *C. graminicola,* it can be targeted to develop specific successful programs to control anthracnose development in maize (Sanz-Martín, 2016).

MATERIALS AND METHODS

New structure of *CgEP3.* RNA evidence of *CgEP3* transcripts was described using RNA-Seq data of ungerminated conidia samples of *C. graminicola* (shared by Dr. Stephan Wirsel, University of Martin Lutero of Halle-Wittenberg, Germany) (unpublished data). To experimentally demonstrate the *CgEP3* structure, multiple overlapped RT-PCRs were designed. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) and treated with DNase and the TURBO DNA-free™ Kit (Ambion, Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using 1 µg of RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT-PCRs were carried out using a DNA polymerase kit (Biotools, Madrid, Spain) following the recommendations of the handbook. Once the *CgEP3* transcript was confirmed, open reading frames were examined to predict the *CgEP3* coding sequence. Bioinformatics analysis was carried out using Geneious Prime 2020 v0.5 (Geneious, 2020). The primers used are listed in Supplementary Table 4.

The tertiary structure of CgEP3. The objective of the hypothetical three-dimensional structure of CgEP3 was achieved through homology modeling, using the crystallized structure of a partial portion of the 5'-methylthionadenosine/S-adenosylhomocysteine nucleosidase from *Streptococcus pneumoniae* (PDB entry: 1ZOS) (V. Singh et al., 2006; Waterhouse et al., 2018), which displays a 28.1 % homology with CgEP3. The modeling was carried out with Modeler v9.24, employing default parameters (Webb & Sali, 2016). The first 17 amino acid residues of the protein, which were recognized as intrinsically disordered, were removed from the three-dimensional structure. The quality analysis of the model was executed by evaluating the B-factor using ResQ (Yang et al., 2016) and Ramachandran plot from the RAMPAGE server (Lovell et al., 2003). Preparation of the model for molecular dynamics was performed using VMD v1.3.9 (Humphrey et al., 1996): and the protein model was embedded in a 5 Å water box, which was ionized with 0.15 mol/L of NaCl. Molecular dynamics was run through NAMD v2.14 (Phillips et al., 2020), using the force fields from CHARMM36m (J. Huang et al., 2017), Langevin dynamic parameters, and a time step of 2 ft/s. A flexible cell was used for the simulation. The simulation was performed for a duration of 10,000,000 steps for 5 ns.

Phylogenetic analysis of CgEP3. A BLASTP search (Agarwala et al., 2016; Altschul et al., 1990; Boratyn et al., 2013) was performed to identify the 10 most similar proteins using *Colletotrichum* proteomes (Supplementary Table 1) with an e-value cutoff of $1e^{-10}$. Proteins were aligned using MAFFT v7.450 (Katoh & Standley, 2013). The alignment was employed to generate a model using hidden Markov models and Hmmer v3.1b2 (Finn et al., 2011). A second search was carried out using the model and Hmmer with proteomes of the graminicola complex and *C. tofieldiae* of the sphaetianum complex (selected as outgroups) (Supplementary Table 1). The 10 most similar proteins to CgEP3 of each species were aligned again using MAFFT v7.450 (Katoh & Standley, 2013). An unrooted phylogenetic tree was constructed using FastTree v2.1.11 (Price et al., 2009). Conserved domains of proteins were predicted using InterProScan (Quevillon et al., 2005). BLASTP, MAFFT, FastTree, and InterProScan are tools that are found in Geneious Prime 2020 v0.5 (Geneious, 2020).

***Colletotrichum* species tree.** To better understand the phylogenetic relationships among species of the genus *Colletotrichum*, a tree was constructed with 21 genomes available (Supplementary Table 1), which represented nine described species complexes (Talhinhas & Baroncelli, 2021). The full sequences of actin (*act*), chitin-synthase 1 (*chs-1*), and 3-phosphate dehydrogenase (*gadph*) genes were used to generate the phylogenetic tree. MAFFT v7.450 (Katoh & Standley,

2013) was used to carry out multiple alignments of sequences that were exported to the free software Mega X (Sudhir Kumar et al., 2018). Subsequently, these data were utilized to calculate a model of best-fit substitutions in each group of sequences separately. Geneious Prime 2020 v0.5 (Geneious, 2020) was deployed to concatenate the alignment of *act, chs-1,* and *gadph* sequences. Next, a phylogenetic tree was constructed with MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003) using a Markov Chain Monte Carlo (MCMC) algorithm and Bayesian interference. Subsequently, Mega X (Sudhir Kumar et al., 2018) was used to create a nucleotide substitution model for each gene and locus. Finally, an analysis of four MCMC chains was carried out as trees that were random through five million generations, and each hundred generations were sampled.

Synteny analysis. The *CgEP3* genomic region was analyzed by selecting the sequence that represented the four genes that were upstream and downstream of *CgEP3,* including *CgEP3.* These genes have been identified in the genomes (Supplementary Table 1) through a TBLASTN search (Agarwala et al., 2016; Altschul et al., 1990; Boratyn et al., 2013). Genomic regions were retrieved for comparison with different tools. The sequences of the selected genomes were aligned using Mauve (Darling et al., 2004). Specifically, the genomic regions of *C. graminicola* and *C. navitas* were aligned with MAFFT v7.450 (Katoh & Standley, 2013). Similarly, synteny analysis was performed between 20 different *C. graminicola* strains (Supplementary Table 2). The *CgEP3* gene was searched for in genomes using BLASTN (Agarwala et al., 2016; Altschul et al., 1990; Boratyn et al., 2013). The sequences were retrieved and aligned using MAFFT v7.450 (Katoh & Standley, 2013) and the alignment was used to construct a phylogenetic tree using FastTree v2.1.11 (Price et al., 2009). TBLASTN, BLASTN, MAFFT, and FastTree are tools that are included in Geneious Prime 2020 v0.5 (Geneious, 2020).

Subcellular localization of CgEP3. *In silico* analysis was conducted with the CgEP3 protein sequence with and without the signal peptide using different online applications (Supplementary Table 3), following the indications of Robin et al. (2018). Heterologous transient expression of *CgEP3* in tobacco plants (*Nicotiana benthamiana*) was based on the work of Robin et al. (2018). Briefly, the *CgEP3* cDNA sequence without its predicted signal peptide was synthesized using GeneART® gene synthesis (Invitrogen, Darmstadt, Germany). Next, the construct was cloned into the donor vector pDONR221 (Invitrogen, Darmstadt, Germany). To transiently express CgEP3 in tobacco plants, the construct was recombined into a pSITE-CA binary vector, where the N-terminus was fused with EGFP. Next, the plasmid construct was cloned in two steps: first in, *Escherichia coli* TOP10 cells, and second in, *Agrobacterium tumefaciens* C58C1. Different transformants were confirmed using PCR with pairs of primers EGFP-SubcL Fw and pCaMV35S-SubcL Rv. *A. tumefaciens* suspension was pressure infiltrated in the abaxial face of tobacco leaves, which were cut into small pieces at 48-72 h after agro-infiltration to investigate using a confocal microscope. The primers used in this experiment are listed in Supplementary Table 4.

Cultivation of fungi and plants. The *C. graminicola* M1.001 wild type strain (L. J. Vaillancourt & Hanau, 1991), together with transformant strains, were grown following the work of Sukno et al. (2008) in solid media, including potato dextrose agar (PDA) (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), minimum agar, and oatmeal agar (Difco) under constant white light at 23 °C. Potato dextrose broth (Difco) was used to grow vegetative mycelia. Phenotypic characterization of the strains was performed through sporulation and growth rate experiments. We followed the protocol of Thon et al. (2002) and Fang et al. (2002). The conidial germination rate assay was adapted from Valèrio et al. (2005). When sporulation occurred, 6

drops of 15 µL of 10^4 conidia/mL were inoculated into Petri dishes containing water agar (2.5%). For germination, the plates were incubated under continuous white light at 23 °C for 24 h. Germination was stopped, and the cells stained by adding a drop of lactophenol blue solution (Sigma-Aldrich, 61335) to the conidia. A coverslip was applied to a blue drop to count germinated conidia under a light microscope. Conidia and appressoria morphology analyses were performed using the slide-culture method (Cai et al., 2009; Ulrike Damm et al., 2013). A small block of PDA (1 cm^2) was placed on a slide and introduced inside a Petri dish that included a wet filter paper (both sterile). The block borders were then inoculated with conidia, and a sterile cover slip was used to cover the block. Fungal conidia and appressoria that grew on the lower parts of the slide and cover slips were analyzed 7 days after inoculation. Microscope preparations were stained with a small drop of lactophenol blue (Sigma-Aldrich, 61335) and photographed using a differential interference contrast microscope (Nikon Optiphot 2 microscope (Nikon Instruments Inc., Melville, NY, USA)). Measurements of conidia and appressoria size were carried out with Piximètre 5.1 (Henriot & Cheype, 2020). Maize plants of the derived inbred line Mo940 (Warren, 1976; Warren et al., 1977) were cultivated until they reached the stage of development V3 (approximately 3 weeks old) at 25 °C under long-day conditions (16 h of light and 8 h of dark) and 80% humidity.

Expression profile of *CgEP3*. Maize leaf infection was carried out following the protocol of Vargas et al. (2012), with a spore concentration of 10^6 sp/mL (described below). Quantitative RT-PCR assay and transcriptional fusion of *CgEP3* were carried out as described by Sanz-Martín (2016). In summary, RNA isolation was performed using the SV Total RNA Isolation Kit (Promega, Madison, WI, USA). cDNA synthesis was performed using the PrimeScript RT Reagent Kit (Takara Bio Inc., Otsu, Japan) from 1 µg of RNA. KAPA SYBR Green qPCR Mix (KAPA Biosystems, Wilmington, MA, USA) was used to carry out the quantitative real-time PCR reaction, with 1 µL of reverse transcription reaction mixture. A StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) was employed with the following program: 1) 95 °C for 5 min, 2) 40 cycles of 95 °C for 10 s, 65 °C for 20 s, and 72 °C for 20 s. Additionally, melting curve analysis was performed using the following program: 95 °C for 10 s, and 65 °C for 15 s, with a temperature incrementation of 0.11°C per second, until 95°C. The normalization of expression values was carried out using *histone 3* and *α-actin* genes from *C. graminicola* (Krijger et al., 2008). StepOnePlus™ Sofware v2.3 (Applied Biosystems, Waltham, MA, USA) was used to calculate the relative expression levels using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Additionally, the 5' promoter region of *CgEP3* (predicted at 1 kb upstream of *CgEP3* CDS) was the C-terminally fused with GFP. The promoter region was amplified with 879FTr Fw and 879FTr Rv primers and cloned into the pENTR/D-TOPO entry clone vector (Invitrogen, Carlsbad, CA, USA) and then recombined into the pKW3 binary destiny vector (Vargas et al., 2016). Clones were checked using PCR with 879FTr Fw and GFP Rv and sequencing. Similarly, transcriptional fusion of *β-tubulin* with GFP was carried out (Sanz-Martín, Pacheco-Arjona, et al., 2016). Primers used in these experiments are listed in Supplementary Table 4.

Generation of *CgEP3* transformant strain. The construct for the deletion of the *CgEP3* gene was produced using the DelsGate methodology (García-Pedrajas et al., 2008) and as described by Sanz-Martín (2016). The 5' and 3' flanks that surrounded the *CgEP3* CDS were amplified using the pair of primers 879-1 and 879-2 (for the 5' UTR flank) and 879-3 and 879-4 (for the 3' UTR flank), and cloned into the pKW1 donor vector, which included a hygromycin resistance gene cassette (*hph*) and a green fluorescent protein cassette (*gfp*) (Vargas et al., 2016). Linearization of the construct was carried out with the I-*Sce*I restriction enzyme (Roche Diagnostics, Basel, Switzerland) and consecutively used for protoplast transformation of the *C. graminicola* M1.001

wild-type strain (Serenella A. Sukno et al., 2008; Thon et al., 2000). Transformants in which homologous recombination occurred correctly were identified using PCR amplification with 879KO Fw, GFP Rv, KAN RDG Fw, and 879 KO Rv primers. To generate the constructs for full complementation, a sequence of 1 kb upstream and downstream of the *CgEP3* CDS was amplified using 879 COMP Fw and 879 COMP Rv primers. The pDONR221 Gateway entry vector (Invitrogen, Carlsbad, CA, USA) was used to clone the construct using the LB Clonase II enzyme (Invitrogen). A second step was conducted to clone the construct into a pKW4 binary destiny vector using the LR Clonase II enzyme (Invitrogen, Carlsbad, CA, USA). It contained the nourseothricin resistance gene (*nat*). Finally, the construct was utilized for protoplast transformation of the Δ*CgEP3-18* strain (Serenella A. Sukno et al., 2008; Thon et al., 2000). Fungal transformant strains were confirmed using PCR with 879 COMP Fw and GFP Rv primers. The partial complementation construct was amplified from a sequence that included the native promotor region (1 kb upstream of *CgEP3* CDS), the partial CDS (only with the sequence that encoded the NP-like domain), and an artificial stop codon, using 879 P-COMP Fw and 879 P-COMP Rv primers. The cloning procedure was similar to that of full complementation. Fungal transformant strains were confirmed using PCR with 879 P-COMP Fw and GFP Rv primers. Finally, a strain that constantly expressed *CgEP3* was generated using the USER™ Friendly cloning methodology (Frandsen et al., 2008). It was regulated by the *gpda* promoter of *Aspergillus nidulans.* The construct was constructed using 879 OE Fw and 879 OE Rv primers and included the *CgEP3* CDS and the terminal region of the gene. The construct was cloned into a pRF-HUE vector that was previously digested with *Pac*I and *Nt.BbvC*I restriction enzymes (New England Biolabs, Ipswich, MA, USA). The pRF-HUE vector contained a hygromycin resistance gene cassette (*hph*). The pRF-HUE construct was used to transform the protoplast of the *C. graminicola* M1.001 wild-type strain (Serenella A. Sukno et al., 2008; Thon et al., 2000). Fungal transformant strains were confirmed using PCR with Pgpda Fw and 879 OE Rv primers. The last three constructs (full and partial complementation and constitutive expression) were integrated into the fungal genome through ectopic recombination. Additionally, sequencing was performed to check the constructs before protoplast transformation. Subsequently, Southern blot assays were performed after transformation. The list of primers used to create the genetic constructs and for verification is summarized in Supplementary Table 4.

Extraction of genomic DNA and Southern blot assay. Genomic DNA was extracted from *C. graminicola* using a modified CTAB protocol (Baek & Kenerley, 1998; Irfan et al., 2016; Murray & Thompson, 1980). Southern blot assays were performed following the protocol described by Sambrook and Russell (2001). First, 10 µg of genomic DNA was digested with the restriction enzymes *Pst*I and *Xho*I (Takara Bio Inc., Otsu, Japan). Then, it was run through a 0.8% (w/v) agarose gel to undergo denaturation. A nitrocellulose membrane (Amersham HyBond-N, GE-HealthCare, Little Chanfont, UK) transference was performed. Probes labeled with digoxigenin (dUTP) were designed and created using a DNA labeling kit (Roche Diagnostic, Basel Switzerland): *CgEP3* 5' UTR-CDS (partial 5' UTR and partial *CgEP3* CDS sequence), hygromycin (*hph* resistance gene sequence), and Nourseothricin (*nat* resistance gene sequence), using the pair of primers 879 5'-CDS SB Fw - 879 5'-CDS SB Rv, Hyg SB Fw - Hyg SB Rv, and Nours SB Fw-Nours SB Rv, respectively. Finally, the probes were hybridized to the membranes. Membrane washes were performed under high-stringency conditions. The primers used are listed in Supplementary Table 4.

Anthracnose pathogenicity assays. Maize leaf infection was carried out following the protocol described by Vargas et al. (2012). Briefly, 3-week-old (state of development V3) maize plants were placed horizontally on a tray containing a moisture paper. The conidia were isolated and

filtered through a double cheesecloth. The conidia were then purified by washing three times with water, and a spore solution of 3×10^5 sp/mL was prepared. Inoculation was carried out on either side of the rib of the third leaf of each maize plant, with 20 drops (10 drops each per side) of 7.5 µL of spore solution. Plants were cultivated for 3–4 days at 25 °C, with a long day cycle (16 h of light and 8 h of dark) and 80% humidity. The maize stem infection protocol was adapted from Eisermann et al. (2019). Inoculation of maize plants of 6-week-old were performed with 5 µL of a spore solution of 2×10^6 sp/mL which was introduced into a small hole (depth of 2-3 mm) made at the first internode of the stalk (just above the adventitious roots). Parafilm was added around the infection point to prevent further infections of the hole. The stalks were divided longitudinally to measure the necrotic areas at 5 dpi. Maize plants were grown under greenhouse conditions (26°C ± 6°C of temperature and 45% moisture). For both experiments, Paint.NET v4.2.16 (dotPDN, LCC) was used to measure the lesion size.

Quantification of fungal biomass. Maize leaf infection was carried out following the protocol of Vargas et al. (2012), with a spore concentration of 10^6 sp/mL (described above). The calculation of the relative amounts of *C. graminicola* wild-type and mutant strains during maize leaf infection is described in the works of Sanz-Martín (2016) and Weihmann et al. (2016). Briefly, maize leaf lesion samples were collected at 3 dpi using a cork-borer with a diameter of 5 mm. Genomic DNA was extracted using the CTAB method (described above). The relative biomass of fungus to maize was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) to analyze differences in DNA templates. The normalization of values was carried out using the internal transcribed spacer 2 (ITS2) of the ribosomal RNA-coding DNA cluster (rDNA) of *C. graminicola* and elongation factor 1 alpha gene (*ZmEF1α*) of maize. qRT-PCR was carried out following the conditions described above, but 10 ng of genomic DNA was used for the reaction template. StepOnePlus™ Sofware v2.3 (Applied Biosystems, Waltham, MA, USA) was used to perform the calculations. The primers used are listed in Supplementary Table 4.

Plant epidermis penetration and papillae apposition assays. Maize plants were infected with *C. graminicola* M1.001 and mutant *CgEP3* strains until 36 hpi, following the protocol of Vargas et al. (2012) with a spore concentration of 10^5 sp/mL (described above). Microscopy was performed through the adaptation of different protocols of Stadnik and Buchenauer (2000) and Fernandez and Heath (1986). Leaf infection points were cut into small pieces that were transferred to a solution of ethanol: glacial acetic acid (3:1, v/v) and incubated for 24 h for tissue bleaching and fixation. If the tissue was not clear, it was necessary to repeat this step for an additional 24 h replacing the solution. Next, the small pieces were placed in distilled water for 4 h and then in lactoglycerol solution (1:1:1, v/v, lactic acid, glycerol, and water) for long-term storage. To stain the samples, it was necessary to remove the lactic acid. Leaf pieces were immersed in glycerol 30% for 1 h and then washed three times with distilled water for 1 h each time. The samples were immersed in 0.05% toluidine blue O (Matheson, Coleman, and Bell) in 0.05 M citrate buffer (sodium citrate dihydrate 0.0352 M, citric acid 0.0148 M), pH 3.5, for 25 h at 4 °C and in darkness. Toluidine blue O was used to remove plant cell autofluorescence. The leaf pieces were then quickly washed with distilled water and immersed in 0.07 M K_2HPO_4, pH 8.9, three times for 1 h each time at ambient temperature (20 °C to 25 °C) and in darkness. Finally, the samples were stained in 0.01 % Aniline blue (water soluble C.I.42755, British Drug Houses Ltd., Canada) in 0.07 M K_2HPO_4, pH 8.9, for at least 2 h at ambient temperature and in darkness. The tissue was observed using light and fluorescence microscopy.

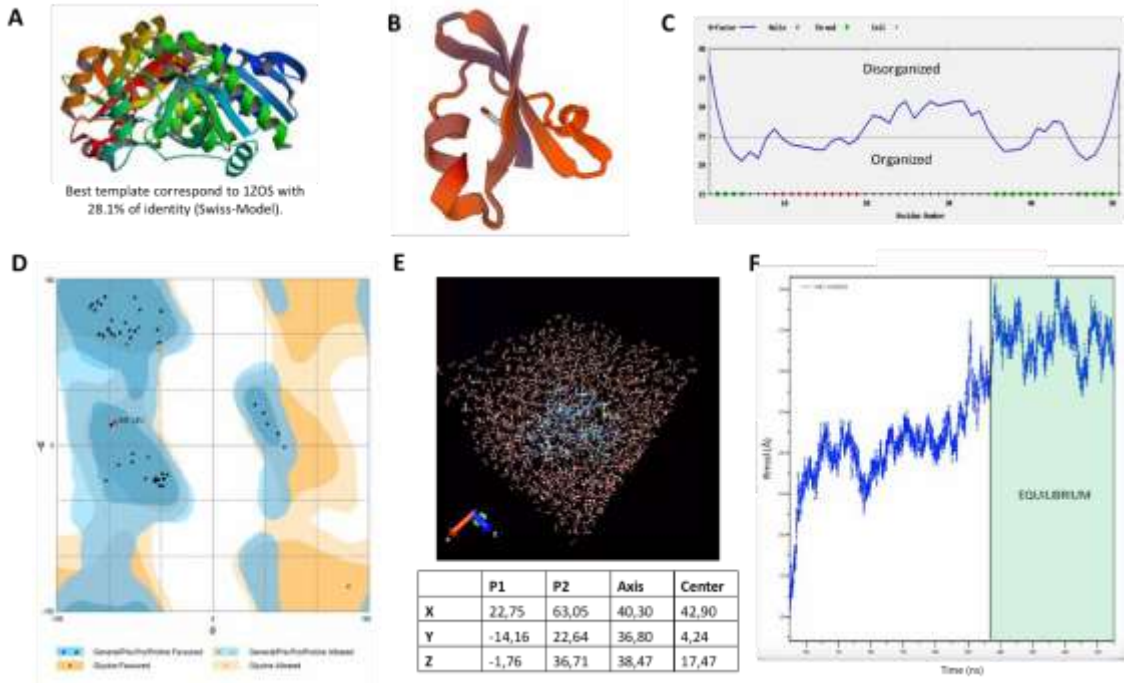
Assessment of appressorial cell wall rigidity and Nile Red staining. To evaluate turgor pressure, polyethylene glycol (PEG) treatment was performed (Howard et al., 1991). This protocol was

adapted from Eisermann et al. (2019). Fresh conidia were collected from PDA Petri dishes in 10 mL of sterile water with a spore concentration of 4×10^6 sp/mL. The spore suspension was transferred onto a plastic sheet (Avery Zweckform Transparant Sheets Laser A4, model 3562. Toronto, Canada), which was placed inside a Petri dish 5.5 cm in diameter. Later, incubation was conducted for 1 h at ambient temperature (20 °C to 25 °C), after which the liquid was discarded. To allow fungal growth, a moist filter paper was placed in a Petri dish lid that was then sealed with parafilm. Growing conditions were maintained at 23 °C for 24 h in the absence of light. PEG 6000 (Sigma-Aldrich, Merck, Darmstadt, Germany) treatments at selected concentrations were carried out through a first wash of plastic sheets with 15 mL of water, and then the sheets were incubated in PEG 6000 for 15 min at ambient temperature. Microscopic preparations were observed using a light microscope. To study appressorial lipid dynamics of fungal strains, appressoria were stained with Nile Red dye (Sigma-Aldrich, Merck, Darmstadt, Germany). This protocol was adapted from Ludwig et al. (2014) and Eisermann et al. (2019). Appressoria were grown as described previously (slide-culture method). Nile Red (500 µg/mL) was dissolved in acetone and appressoria added and incubated for 30 min. Then, they were washed with phosphate-buffered saline buffer (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, and 135 mM NaCl, pH 7.4) for 10 min. This step was repeated three times.

Microscopy and Image Processing. An inverted confocal microscope (Leica SP5, Leica Microsystems GmbH, Germany) was used to determine the subcellular localization of CgEP3 in *N. benthamiana.* A Dragonfly 200 spinning disk confocal laser microscope (Andor Inc., Belfast, UK) was used to obtain transcriptional fusion micrographs. In both cases, GFP fluorescence was photographed using a laser line with an excitation wavelength of 488 nm and an emission wavelength of 490–525 nm. Differential interference contrast microscopy was performed using a Nikon Optiphot 2 microscope (Nikon Instruments Inc., Melville, NY, USA). Appressorial penetration assays were performed using a Leica DM5500B light and epifluorescence microscope (Leica Microsystems, Wetzlar, Germany). Micrographs were taken using ultraviolet illumination and a blue filter (excitation BP 360/40 nm and emission BP 470/40 nm). The rest of the experiments were performed with bright field and fluorescence microscopy using a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany). To image appressorial lipid vesicles, the N2.1 filter was used with an excitation wavelength of 515–560 nm and an emission wavelength of 590 nm. Micrographs were processed using the free open-source image-processing platform ImageJ/Fiji (Abràmoff et al., 2004; Schindelin et al., 2012).

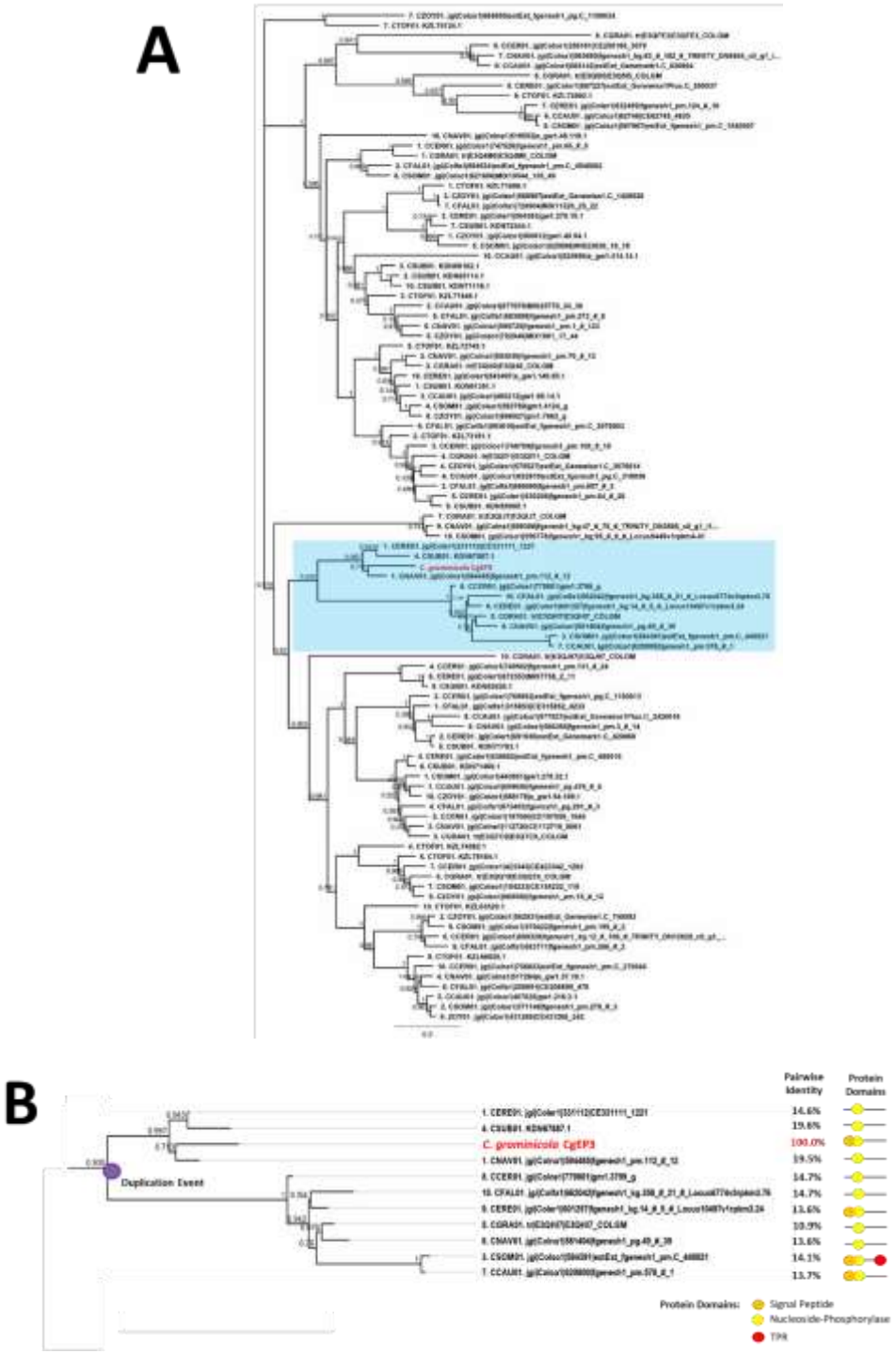
Statistical analysis. Statgraphics Centurion XVII (Statgraphics Technologies, Inc.) was used to perform the statistical tests. Different statistical tests have been used for the comparison of averages. If the data population followed a normal distribution and the variance between the data populations was homogeneous, parametric tests were used, such as one-way ANOVA followed by Tukey's HSD or unpaired *t* test. For data populations that did not follow these assumptions, a nonparametric test such as the Mann-Whitney-Wilcoxon test was utilized. After performing the statistical test, the P value was checked to define if there were statistically significant differences in the averages of the populations ($P < 0.05$ or 0.01).

SUPLEMENTARY INFORMATION

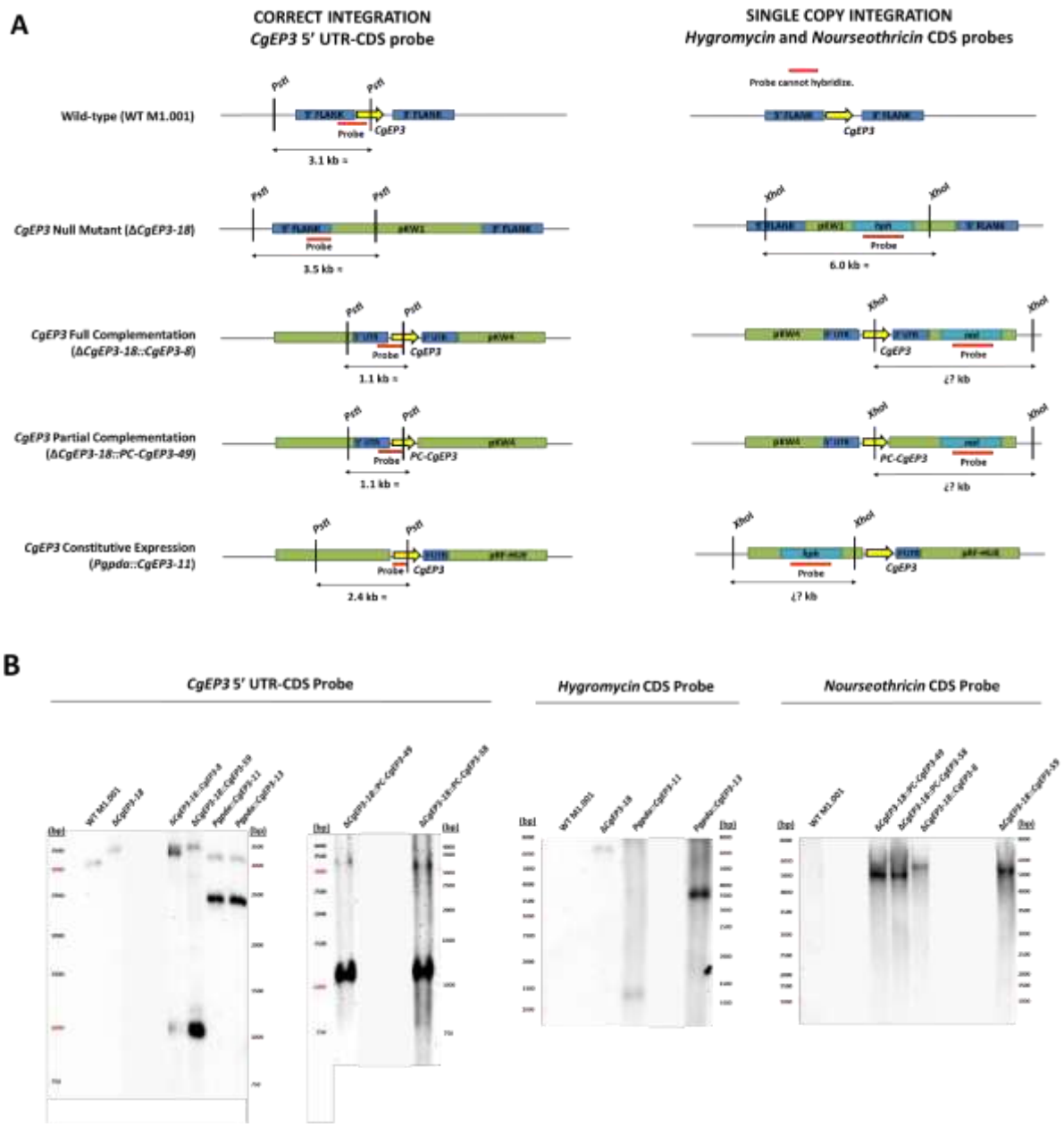


	P1	P2	Axis	Center
X	22,75	63,05	40,30	42,90
Y	-14,16	22,64	36,80	4,24
Z	-1,76	36,71	38,47	17,47

Supplementary Figure 1. Realization of CgEP3 Three-dimensional model. A) Based on homology modeling method, we looked for the best protein model template in Swiss-Model (Waterhouse et al., 2018) which matched with 1ZOS that has a 28.1% of identity with CgEP3. 1ZOS model corresponded with the structure of 5'-methylthionadenosine/S-Adenosylhomocysteine nucleosidase from *Streptococcus pneumoniae* (Berman et al., 2000; V. Singh et al., 2006). **B)** We performed a putative CgEP3 model through homology of 1ZOS using the software Modeller v9.24 (Webb & Sali, 2016). The first 17 amino acid residues of the protein which are recognized as intrinsically disordered were removed of the three-dimensional structure. **C)** CgEP3 model was checked through B-Factor by using ResQ (Yang et al., 2016). B-Factor shows that CgEP3 is stable. N- and C- terminal regions of the protein form secondary structures as alfa helix and beta strands while the central region was disorganized. **D)** Ramachandran Plot quality control (Lovell et al., 2003). The plot represents: in dark blue, Pro-Pro favored regions; in light and dark orange, the allowed Pro-Pro regions; and in white, the disallowed regions. The plot demonstrates that the sum of favored and allowed regions is 97.9%, then its quality is optimal. **E)** Preparation of the model for molecular dynamic was done with VMD v1.3.9 (Humphrey et al., 1996). Above, CgEP3 model was embedded in a water box of 5 Å of size, ionized with 0.15 mol/L and it has also included hydrogen bonds that can form in the protein. Below, we calculated tridimensional coordinates (X, Y, Z) of the sides (P1 and P2), the axis, and the center of the protein model inside water box measured in Armstrong. **F)** Molecular dynamics was run through NAMD v2.14 (Phillips et al., 2020) using different physicochemical and quantic parameters: force fields from CHARMM36m (J. Huang et al., 2017) the Langevin Dynamic parameters, and a time step pf 2 ft/s. A flexible cell was used during the simulation which was performed in a duration of 10,000,000 steps by 5 ns. Evolution of time of backbone atoms of protein model was represented trough the root-mean-square deviation (RMSD). Due to the atom coordinates shows a constant and continuous behavior in the time, the protein model gets to equilibrium (3 ns approximately). Then, the predicted model of CgEP3 is optimal and valid.



Supplementary Figure 2. CgEP3 has low similarity with other proteins of species of *Colletotrichum* genus. A) Phylogenetic tree based on the search of ten best similar proteins to CgEP3 through software Hmmer v3.1b2 (Finn et al., 2011) and Hidden Markov Models of proteomes of the most related species to *C. graminicola* (CGRA01) (red) in graminicola and acutatum complexes: *C. cereale* (CCER01), *C. eremochloae* (CERE01), *C. sublineola* (CSUB01), *C. falcatum* (CFAL01), *C. navitas* (CNAV01), *C. caudatum* (CCAU01), *C. somersetense* (CSOM01) *and C. 104egión104* (CZOY01). As outgroup we selected *C. tofieldiae* (CTOF01) of spaethianum complex. Blue area represented the cluster where CgEP3 was found. **B)** Focus area of blue cluster. It is represented pairwise identity of CgEP3 against each protein and the conserved domains found in its structure. All proteins conserved NP domain (yellow). The protein with the greatest similarity belongs to *C. navitas* (19.5%). There are other proteins that has a SP (orange) that overlap with NP domain in *C. eremochloae* (9. CERE01), *C. somersetense* (3. CSOM01), and *C. caudatum* (7. CCAU01). Because proteins had low similarity compared with CgEP3, we think that it is specific of specie *C. graminicola.* In addition, it is possible that CgEP3 was originated from ancestral gene duplication (purple) occurring during the evolution of this fungi. This duplication could give two groups of proteins where is included CgEP3.



Supplementary Figure 3. Southern blot analysis checking of *CgEP3* transformant strains. A) Scheme of planification of Southern blot assay. On the left, we checked the integration of constructs in *C. graminicola* genome through a probe that hybridized in *CgEP3* 5' UTR and CDS partially. Genomic DNA samples were digested with *Pst*I restriction enzyme. It is showed expected sizes after digestion. On the right, we checked single copy integration of the constructs in the genome of *C. graminicola* thanks to two probes which hybridized in *hygromycin* and *nourseothricin* CDS resistance genes. Genomic DNA samples were digested with *Xho*I restriction enzyme. Probes could not hybridize in wild type. Null mutant strain was the only construct that we expected a size because it is produced by homologous recombination. The rest, we did not know the size because they were integrated ectopically into genome. **B)** On the left, southern blot analysis of the constructs that were correctly integrate into genome. *CgEP3* 5' UTR-CDS probe hybridized in wild-type and null mutant strains with a difference of size of 0.4 kb approximately. In full and partial complementation strains, probe could hybridize twice: in null mutant construct and either full or partial complementation construct respectively. In the same way, probe could hybridize two times in constitutive expression strain: in native *CgEP3* and constitutively expression construct. Because we obtained expected sizes, constructs were correctly integrated in genome. On the right, southern blot analysis of single copy integration. We expected that probes hybridize once in the genomes of transformant strains and results showed this fact. Then we conclude that they had only one copy integration. The probes could not hybridize in wild type strain because it did not have antibiotic resistance genes in its genome.

Supplementary Table 1. List of sequences genomes and derived proteomes used to perform phylogenetic analysis. In order: name of the specie; isolate; species complexes based on (Bhunjun et al., 2021); accession number, BioProject number and BioSample number; and references. (*) JGI means Joint Genome Institute (Grigoriev et al., 2012; Nordberg et al., 2014).

Specie	Isolate	Complex	Accession number	BioProject	BioSample	Reference
C. orbiculare	MAFF 240422	Orbiculare	AMCV00000000.1	PRJNA171217	SAMN02981452	Gan et al., 2013
C. plurivorum	LFN00145	Orchidearum	WIGO00000000.1	PRJNA577396	SAMN13025176	Rogério et al., 2020
C. truncatum	CMES 1059	Truncatum	VUJX01000000.1	PRJNA563581	SAMN12683944	Rogério et al., 2020
C. fructicola	CGMCC3.17371	Gloeosporioides	SSNE00000000.1	PRJNA532407	SAMN11398962	Armitage et al., 2020
C. chlorophyti	NTL11	None	MPGH00000000.1	PRJNA350752	SAMN05945917	Gan et al., 2017
C. higginsianum	IMI 349063	Destructivum	LTAN00000000.1	PRJNA47061	SAMN04420120	Zampounis et al., 2016
C. tofieldiae	0861	Sphaetianum	LFIV00000000.1	PRJNA286731	SAMN03769761	Hacquard et al., 2016
C. incanum	MAFF 238704	Sphaetianum	LFIW00000000.1	PRJNA286717	SAMN03769590	Hacquard et al., 2016
C. cereale	CBS 129662	Graminicola	NA	PRJNA262440	SAMN05660427	JGI (*)
C. eremochloae	CBS 129661	Graminicola	NA	PRJNA262442	SAMN05660823	JGI (*)
C. sublineola	TX430BB	Graminicola	JMSE00000000.1	PRJNA246670	SAMN02769489	Baroncelli et al., 2014b
C. falcatum	MAFF 306170	Graminicola	NA	PRJNA262221	SAMN05660685	JGI (*)
C. graminicola	M1.001	Graminicola	ACOD00000000.1	PRJNA225514	SAMN02953757	O'Connell et al., 2012
C. navitas	CBS 125086	Graminicola	NA	PRJNA262371	SAMN05660908	JGI (*)
C. caudatum	CBS 131602	Caudatum	NA	PRJNA262368	SAMN05660677	JGI (*)
C. somersetense	CBS 131599	Caudatum	NA	PRJNA262441	SAMN05660317	JGI (*)
C. zoysiae	MAFF 235873	Caudatum	NA	PRJNA262217	SAMN05660351	JGI (*)
C. orchidophilum	IMI 309357	None	MJBS00000000.1	PRJNA342923	SAMN05771038	Baroncelli et al., 2018
C. godetiae	CBS 193.32	Acutatum	NA	PRJNA333328	SAMN05446267	JGI (*)
C. fioriniae	IMI 504882	Acutatum	JARH00000000.1	PRJNA233987	SAMN02580179	Baroncelli et al., 2014ª
C. lupini	IMI 504893	Acutatum	CP019471-82	PRJNA360503	SAMN06211573	JGI (*)

Supplementary Table 2. List of genomes sequenced before in our group of different *C. graminicola* isolates of the world used to carry out phylogenetic analysis. Name of the isolate and isolation origin. The data have not still published.

Specie	Isolate	Isolation place
C. graminicola	M1.001	USA
C. graminicola	DMSZ63127	Germany
C. graminicola	LARS318	Nigeria
C. graminicola	CBS113173	Zimbabwe
C. graminicola	NRRL47511	USA
C. graminicola	NRRL13649	USA
C. graminicola	CRO-I-35	Croatia
C. graminicola	F40300-1	France
C. graminicola	CR10370-50	Croatia
C. graminicola	F64330-7	France
C. graminicola	SW8046-2	Switzerland
C. graminicola	CBS 252.59	Netherlands
C. graminicola	F64330-2	France
C. graminicola	SW8046-1	Switzerland
C. graminicola	P7565-07212	Portugal
C. graminicola	CA-CHAT-1	Canada
C. graminicola	CRO-I-41	Croatia
C. graminicola	CA-N8H	Canada
C. graminicola	ARG2700-2	Argentina
C. graminicola	M5.001	Brazil

Supplementary Table 3. *In silico* analysis of CgEP3 subcellular localization. We have used different online applications to run with either full sequence of protein (SignalP v5.0 and Predotar v1.4) or the rest without signal peptide (following the indications of Robin et al., 2018. In some cases, it run with specific mode. The score value represents the probability of prediction: values >0.8 represents a confident prediction, 0.5-0.8, medium confident, and <0.5, low confident. In all cases of the table, NA means non-available. Results showed that CgEP3 is an effector, without transmembrane domains, secreted and it could have nuclear localization. It was not predicted nuclear or nucleolar localization signal in its structure. There were other predictions but with low probability.

Application	Reference	Protein Sequence	Mode	CgEP3 Subcellular Prediction	Score
SignalP v5.0	Armenteros et al., 2019ª	Full sequence	Eukarya	Signal Peptide	0.88
ApoplastP	Sperschneider et al., 2018	Without SP	NA	Non apoplastic	0.63
TMHMM Server v2.0	Krogh et al., 2001	Without SP	NA	Non-transmembrane	1.00
TargetP v1.1 Server	Armenteros et al., 2019b	Without SP	Plant	Other	0.64
EffectorP v1.0	Sperschneider et al., 2016	Without SP	NA	Efector	0.95
LOCALIZER v1.0.4	Sperschneider et al., 2017	Without SP	Effector	Non chloroplast, mitochondria, nucleus	NA
ChloroP v1.1 Server	Emanuelsson et al., 1999	Without SP	NA	Chloroplast	0.43
Predotar v1.4	Small et al., 2004	Full sequence	Plant	Possibly ER	0.35
Yloc	Briesemeister et al., 2010	Without SP	Plant	Secretory Pathway	0.95
BaCelLo	Pierleoni et al., 2006	Without SP	Plant	Secretory	NA
WoLFP SORT	Horton et al., 2007	Without SP	Plant	Nucleus	NA
Predict NLS	Cokol et al., 2000	Without SP	NA	No	NA
Predict DNA Binding Residues	Cokol et al., 2000	Without SP	NA	No	NA
cNLS Mapper	Kosugi et al., 2009	Without SP	NA	No	0.70
NLS Tradamus	Nguyen Ba et al., 2009	Without SP	NA	No	0.70
Nucleolus NoD	Scott et al., 2011	Without SP	NA	No	0.80
EuK-mPLoc v2.0	Chou and Shen, 2010	Without SP	NA	Extracellular	NA

Supplementary Table 4. List of primers used in this study. They were designed with NetPrimer (Premier Biosoft) and obtained from IDT (Integrated DNA Technology Inc., Coralville, IA, USA).

Primer name	Sequence 5' – 3'	Description	Reference
879 RT-A Fw	CCTCACTATTCCATACCTCACCAC	*GLRG_00879* RT-PCR – A	This article
879 RT-A Rv	CGTCGCCACACTTTTGC	*GLRG_00879* RT-PCR – A	This article
879 RT-B Fw	TGTGTCTGGTGGTAGCAAAGTG	*GLRG_00879* RT-PCR – B	This article
879 RT-B Rv	CGAAGGGCTCTAACGGTG	*GLRG_00879* RT-PCR – B	This article
879 RT-C Fw	CCTCACTATTCCATACCTCACCAC	*GLRG_00879* RT-PCR – C	This article
879 RT-C Rv	AGGCAACCTTTCTGTATTCAG	*GLRG_00879* RT-PCR – C	This article
879 RT-D Fw	TTCTGTCGGTGATACGGC	*GLRG_00879* RT-PCR – D	This article
879 RT-D Rv	CGAAGGGCTCTAACGGTG	*GLRG_00879* RT-PCR – D	This article
879 RT-E Fw	TGATAGGTCGCATTTGAGGA	*GLRG_00879* RT-PCR – E	This article
879 RT-E Rv	TCGTCGGTTGTGATTGATTCT	*GLRG_00879* RT-PCR – E	This article
879 RT-F Fw	GCACCGTTAGAGCCCTT	*GLRG_00879* RT-PCR – F	This article
879 RT-F Rv	GCGGATCTGCGACTC	*GLRG_00879* RT-PCR – F	This article
879 RT-G Fw	GGCATCTACGGAACTACCTCG	*GLRG_00879* RT-PCR – G	This article
879 RT-G Rv	GCACACCAGTCCACCTCTAAG	*GLRG_00879* RT-PCR – G	This article
879 RT-H Fw	CTCTTCGTCTTCGTTAGCG	*GLRG_00879* RT-PCR – H	This article
879 RT-H Rv	GAGCAAGTGAAACTCGTATT	*GLRG_00879* RT-PCR – H	This article
879 PCR-I Fw	GACTGCTGCGAACTGAAAAG	*GLRG_00879* RT-PCR – I	This article
879 RT-I Rv	GCCCACTGCCATCCTTC	*GLRG_00879* RT-PCR – I	This article
879 RT-J Fw	TCTCCCTGCCTTTCTCGTCC	*GLRG_00879* RT-PCR – J	This article
879 RT-J Rv	TGCCATCAATACAGCATCGT	*GLRG_00879* RT-PCR – J	This article
879 RT-N1 Fw	GCCTGAGCCTGCGTTTTAT	*GLRG_00879* RT-PCR – Negative Control 1	This article
879 RT-N1 Rv	GCTTGCGGCACATTATGA	*GLRG_00879* RT-PCR – Negative Control 1	This article
879 RT-N2 Fw	TCTCCCTGCCTTTCTCGTCC	*GLRG_00879* RT-PCR – Negative Control 2	This article
879 RT-N2 Rv	CCGACCTTCATCGTCTTTCTC	*GLRG_00879* RT-PCR – Negative Control 2	This article
879 RT-Fw	GTTAATTAACCTGAATATACAATGACCC	*GLRG_00879* RT-PCR – Positive Control	Sanz-Martín, 2016
879 RT-Rv	CTCATGACGATCTTTTGGGCCCGGGCG	*GLRG_00879* RT-PCR – Positive Control	Sanz-Martín, 2016
CgTub Fw	CAGTCCCTTGGGCGGCACAG	*C. graminicola β-Tubuline* RT-PCR – Positive Control	Vargas et al., 2016

CgTub Rv	CCCGGGGCAATTGAACGCC	*C. graminicola β-Tubuline* RT-PCR – Positive Control	Vargas et al., 2016
M13 Fw	GTAAAACGACGGCCAG	Confirmation of Gateway entry clone	Invitrogen, 2012
M13 Rv	CAGGAAACAGCTATGAC	Confirmation of Gateway entry clone	Invitrogen, 2012
EGFP – SubcL Fw	TCAAGGAGGACGGCAACATC	Amplification of EGFP Cassette	This article
pCaMV35S – SubcL Rv	TGAGCGAAACCCTATAAGAACCC	Amplification of pCaMV35S	This article
Cg879 qPCR Fw	GCCAGATCCAGGGACATAAC	qPCR *GLRG_00879*	Sanz-Martín, 2016
Cg879 qPCR Rv	TCGTCGGTTGTGATTGATTCT	qPCR *GLRG_00879*	Sanz-Martín, 2016
CgH3 qPCR Fw	CGAGATCCGTCGCTACCAGA	qPCR *C. graminicola Histone 3*	Krijger et al., 2008
CgH3 qPCR Rv	GGAGGTCGGACTTGAAGTCCT	qPCR *C. graminicola Histone 3*	Krijger et al., 2008
CgACT qPCR Fw	TCCTACGAGCTTCCTGACGG	qPCR *C. graminicola α-actin*	Krijger et al., 2008
CgACT qPCR Rv	CCGCTCTCAAGACCAAGGAC	qPCR *C. graminicola α-actin*	Krijger et al., 2008
879FTr Fw	CACCATCGCCGATACCACACGCCT	Amplification of *GLRG_00879* promoter region	Sanz-Martín, 2016
879FTr Rv	TCAATAGTCGAAGGGCTCTAACG	Amplification of *GLRG_00879* promoter region	Sanz-Martín, 2016
TubFus Fw	CCCGCTACTCAAGACAGGTGGT	*C. graminicola* β-tubuline transcriptional fusion	Sanz-Martín et al., 2016ª
TubFus Rv	CACCAGCTTCCTGCCCCCACGGTTAT	*C. graminicola* β-tubuline transcriptional fusion	Sanz-Martín et al., 2016ª
879-1	TAGGGATAACAGGGTATTATCGCCGAATC CACACGCCT	Amplification of 5´flanking 108egión of *GLRG_00879*	Sanz-Martín, 2016
879-2	GGGGACAAGTTTGTACAAAAAAGCAGGC TAACGAAGGGCTCTAAC	Amplification of 5´flanking 108egión of *GLRG_00879*	Sanz-Martín, 2016
879-3	GGGGACCACTTTGTACAAGAAAGCTGGG TAGCAGATCCGCGGGCT	Amplification of 3´ flanking region of *GLRG_00879*	Sanz-Martín, 2016
879-4	ATTACCCTGTTATCCCTATGAACGCTATCT CGGCAGGCT	Amplification of 3´ flanking region of *GLRG_00879*	Sanz-Martín, 2016
879KO Fw	GGTCTTTGGTTACGGACGGGGA	Confirmation of *CgEP3* deletion	Sanz-Martín, 2016
879KO Rv	GGCTTGATCGCTCTGGTAGC	Confirmation of *CgEP3* deletion	Sanz-Martín, 2016
KAN RDG Fw	ATCGCGGCCTCGACGTTTCC	Amplification of *Kanamycin* cassette	García-Pedrajas et al., 2008
GFP Rv	AAGTCGTGCTGCTTCATGTG	Amplification of *GFP* cassette	Sanz-Martín et al., 2016ª
879 5'-CDS SB Fw	CAATCCTGGCTGCGTTTGC	5'UTR-CDS *GLRG_00879* cassette for Southern blot probe	This article
879 5'-CDS SB Rv	ATAACTATTGTGGTCGGCGGC	5'UTR-CDS *GLRG_00879* cassette for Southern blot probe	This article
Hyg SB Fw	GATCGACGTTAACTGATATTGAAGGAG	*Hygromycin* cassette for Southern blot probe	Sanz-Martín, 2016
Hyg SB Rv	CTATTCCTTTGCCCTCGGACGAGTGCT	*Hygromycin* cassette for Southern blot probe	Sanz-Martín, 2016
Nours SB Fw	TCTTGACGACACGGCTTACC	*Nourseothricin* cassette for Southern blot probe	This article
Nours SB Rv	CATCCACGGGACTTGAGACC	*Nourseothricin* cassette for Southern blot probe	This article
879 COMP Fw	GGGGACAAGTTTGTACAAAAAAGCAGGC TTAGCAGCTATCATGTGTCTGGTGG	*GLRG_00879* cassette for complementation	This article
879 COMP Rv	GGGGACCACTTTGTACAAGAAAGCTGGG TATTCAGCCCACTGCCATCCTTC	*GLRG_00879* cassette for complementation	This article
879 P-COMP Fw	GGGGACAAGTTTGTACAAAAAAGCAGGC TTAGCAGCTATCATGTGTCTGGTGG	*GLRG_00879* cassette for partial complementation	This article
879 P-COMP Rv	GGGGACCACTTTGTACAAGAAAGCTGGG TCCTAAGGTAGTTCCGTAGATGCCTGC	*GLRG_00879* cassette for partial complementation	This article
879 OE Fw	GGACTTAAUTGAATATACAATGACCCATG CCCAGCA	*GLRG_00879* cassette for constitutive expression	This article
879 OE Rv	GGGTTTAAUCACCAGTCCACCTCTAAGGA CCAA	*GLRG_00879* cassette for constitutive expression	This article
PgpdA Fw	GTTGACAAGGTCGTTGCGTCAGT	Amplification of *PgpdA* promotor	Vargas et al., 2016
Cg ITS2 Fw 1.1	CGTCGTAGGCCCTTAAAGGTAG	qPCR *C. graminicola* ITS2	Weihmann et al., 2016
Cg ITS Rv 1	TTACGGCAAGAGTCCCTC	qPCR *C. graminicola* ITS2	Weihmann et al., 2016
Zm EF1α Fw	TGGGCCTACTGGTCTTACTACTGA	qPCR *Zea mays* elongation factor 1 α	Lin et al., 2014
Zm EF1α Rv	ACATACCCACGCTTCAGATCCT	qPCR *Zea mays* elongation factor 1 α	Lin et al., 2014

CHAPTER II:

RNA-Seq analysis of CgEP3 during early stages of maize infection by the phytopathogenic fungus *Colletotrichum graminicola*.

ABSTRACT

The phytopathogenic fungus *Colletotrichum graminicola* (Ces.) Wils causes the disease anthracnose in maize. *Colletotrichum graminicola* secretes effector proteins to regulate the host immune system. Here, we studied *C. graminicola* effector protein 3 (CgEP3), which is specific to *C. graminicola.* CgEP3 plays a role in virulence during the early stages of infection of maize and can facilitate the fungus to penetrate the epidermis of the host plant. To better understand the function of CgEP3 during leaf infection, we performed transcriptional profiling using RNA sequencing. The transcriptomic results revealed 56 differentially expressed genes (DEGs) in maize, 35 of which may be suppressed by CgEP3. These DEGs are involved in abscisic acid signaling, chlorophyll biosynthesis, processes associated with photosystems, electron transport chain, transcription factors such as MYB and CO-Like/BBX metabolism of selenium, carbon (through glycolysis), and lipids (through phytosterol biosynthesis), secondary metabolism (through the phenylpropanoid pathway), RNA metabolism; protein folding and degradation, and plant cell remodeling. The suppression of these pathways by CgEP3 can counteract plant defenses, enabling the colonization and development of disease. On the contrary, 21 of the 56 DEGs could be induced by CgEP3 and were found to be associated with pathogenesis-related proteins; ethylene signaling; and gibberellin biosynthesis; transcription factors such as WRKY, GT (trihelix), and TPL; sulfur and iron metabolism; and secondary metabolism (through the sesquiterpenoid phytoalexin pathway). The induction of these pathways is attributed to the fact that maize can recognize CgEP3 and consequently activate the expression of these genes to improve plant defense. Taken together, these genes represent several independent defense mechanisms that could be suppressed or induced by the CgEP3 effector at the early stages of infection.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most widely cultivated crops around the world and is also favored as a model species for research (Sanz-Martín, Pacheco-Arjona, et al., 2016; Strable & Scanlon, 2009; Wu & Guclu, 2013). The fungal genus *Colletotrichum* contains phytopathogens which are found globally (R. Dean et al., 2012; Gorman et al., 2020) and are considered among the top ten major disease producers of the world. This genus is also used as a model for studying hemibiotrophic plant pathogens (R. Dean et al., 2012; Gong et al., 2020). Members of *Colletotrichum* produce anthracnose disease throughout tropical, subtropical, and temperate zones in a wide variety of dicots and monocots (Bhat et al., 2019a; Cannon et al., 2012; Crous et al., 2004) consisting of significant agricultural crops, such as soybean (*Glycine max*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), and maize (J. A. Bailey, 1992; D. Prusky et al., 2000; Vargas et al., 2016). *Colletotrichum graminicola* (Ces.) Wils is a pathogen that produces the symptoms of leaf blight and stalk rot in maize (Bergstrom & Nicholson, 1999; Jamil & Nicholson, 1991; Sanz-Martín, Pacheco-Arjona, et al., 2016) leading to huge amount of losses (up to one billion dollars in the USA) (T. J. Frey et al., 2011; Sanz-Martín, Pacheco-Arjona, et al., 2016). The infection of maize leaves starts when a spore lands on the leaf surface and germinates. This process occurs approximately within 12 h after the spore comes in contact with the host plant. Throughout the next 24 h, the spore develops a germ tube at whose tip a melanized, dome-shaped appressorium is formed. Subsequently, the appressorium generates a penetration peg that penetrates the cell wall of the host plant. *Colletotrichum graminicola* then begins to invade the host cells (approximately 24–36 h after the spore comes in contact with the host plant. When penetration is successful, bulbous primary hyphae start to colonize host cells

and this phase is known as biotrophy (Gong et al., 2020; Gorman et al., 2020; Kleemann et al., 2008; Ludwig et al., 2014; Mims & Vaillancourt, 2002; R. J. O'Connell et al., 2012; D. Politis & Wheeler, 1972; Vargas et al., 2012, 2016). Primary hyphae with irregular shapes continue growing inside the plant cells and start to form branches that spread to other adjacent cells. At this stage of the disease, the fungus maintains live plant cells and does not produce any discernible symptoms (Gong et al., 2020; Micali et al., 2011; R. J. O'Connell et al., 2012; Panstruga, 2003; Perfect & Green, 2001; Sanz-Martín, Pacheco-Arjona, et al., 2016). Finally, the fungus changes its behavior and begins to form thin secondary hyphae approximately 60 to 72 h after the spore first comes in contact with the host plant. This is known as the necrotrophic phase of the disease, where *C. graminicola* kills the host cells and necrotic lesions start to form on the infected tissues (Bergstrom & Nicholson, 1999; Gorman et al., 2020; Mims & Vaillancourt, 2002; R. J. O'Connell et al., 1985; Vargas et al., 2012; P. S. Wharton et al., 2001).

To develop a compatible host-pathogen interaction, pathogens have to break the different plant defenses, such as structural and chemical barriers (De Jesus Miranda et al., 2017; Eisermann et al., 2019; Jones & Dangl, 2006; Sels et al., 2008). Host plants can recognize specific structures of the fungal pathogens, such as β-glucans, ergosterol, and chitin, which are known as pathogen-associated molecular patterns or PAMPs. These PAMPs can trigger the first layer of the basal plant defense response, the PAMP-triggered immunity (PTI) (Eisermann et al., 2019; Lochman & Mikes, 2006; Oliveira-Garcia & Deising, 2013; Wan et al., 2008) which involves several changes, such as cell wall restructuring, papillae deposition, generation of reactive oxygen species, biosynthesis of phytoalexins, and production of pathogenesis-related proteins (PRs) (Eisermann et al., 2019; Newman et al., 2013; Vargas et al., 2016). The fungi adapt to the internal conditions of the host to prevent the PTI defense response from being triggered. They can release effectors that help the them to not only escape the PTI response, but also compromise it, facilitating the development of the disease (Eisermann et al., 2019; Oliveira-Garcia & Valent, 2015). However, occasionally, the host plants can recognize effectors through certain proteins that trigger the second layer of defense, the effector-triggered immunity (ETI) response. This is more robust than the PTI and checks the progression of the pathogen (Jones & Dangl, 2006; Vargas et al., 2016). In some cases, a hypersensitive response occurs as a result of ETI. This behavior is typically observed in plants infected with race-specific pathogens. ETI produces necrosis at the infection site resulting in cell death. Meanwhile, lignin and phenolic compounds are deposited in the cell wall at the infection point, causing its hardening (Bhat et al., 2019a; Garcia-Brugger et al., 2006). *Colletotrichum graminicola* can release effector proteins to improve the process of infection (Eisermann et al., 2019). Overall, effectors can be defined as proteins that are secreted by pathogens to promote host cell colonization, either interference or manipulation, as well as to protect the fungi from plant defenses (Cook et al., 2015; Fouché et al., 2018). Effectors can target host plant cells to translocate inside and arrive at diverse internal cellular locations, such as cytoplasm, chloroplast, mitochondria, nucleus, nucleolus, and other subcellular locations (Petre et al., 2015; Robin et al., 2018; Vargas et al., 2016; J. Win et al., 2012). Although few studies have been conducted on effectors in *C. graminicola* (Eisermann et al., 2019; Gong et al., 2020; Sanz-Martín, Pacheco-Arjona, et al., 2016; Tsushima et al., 2021; Vargas et al., 2016), further research is required to know how effectors suppress host defenses. One way is to increase the characterization of effectors to determine the role each of them plays during infection (Eisermann et al., 2019).

Different methods have been implemented to comprehensively analyze plant-pathogen interactions. RNA sequencing (RNA-Seq) with differential network analysis is one of the most

popular methods for the task (Bhat et al., 2019a; Pathania et al., 2016). To the best of our knowledge, only a few transcriptome analyses exist related to the defense mechanism in maize in response to *C. graminicola* attack. Tang et al., (2006) used microarrays to analyze the gene expressions during maize infection by *C. graminicola.* O'Connell et al. (2012) used Illumina RNA sequencing to determine the genetic response of fungi during host infection. In a study by Vargas et al., 2012, two subtractive suppression hybridizations were prepared to identify maize differentially expressed genes (DEGs) during the progression of infection. Oliveira-Garcia & Deising (2013), analyzed the gene *GLS1* of maize, which encodes β-1,3-glucan synthase, through RNA-Seq assays to study the regulation of the immunity responses triggered by β-1,3-glucan. Schliebner et al. (2014) utilized RNA-Seq analysis to identify models of genes and events of alternative splicing in *C. graminicola.* Torres et al., 2016, identified *C. graminicola*-specifically expressed genes during the critical phases of host penetration and biotrophic establishment through RNA-Seq. De Jesus Miranda et al. (2017), also used the RNA-Seq approach to search for DEGs involved in defense signaling in maize that are activated locally and systemically during fungal infection. Bhat et al. (2019), attempted to understand the defense responses of maize against *C. graminicola* by employing the RNA-Seq analysis.

Similarly, there are few studies that have used transcriptome analysis to determine how fungal effectors can specifically modulate the defense responses in maize plant. These studies are based on the biotrophic fungal model *Ustilago maydis,* which produces smut disease in maize (Schurack et al., 2020). Doehlemann et al., (2009) analyzed a mutant strain of the fungal effector Pep1 which is incapable of establishing a biotrophic interaction. Through microarray assays, they identified several maize DEGs in the host plants infected with the *U. maydis* effector mutant strain (SG200Δ*pep1*) that are not detected in the wild-type maize. Some of these DEGs were associated with host plant defense. Tanaka et al. (2014), studied the Tin2 fungal effector, which has a minor role in tumor formation. Their microarray analysis determined that the Tin2 effector from *U. maydis* acts as transcriptional inducers for the anthocyanin pathway. Brefort et al. (2014), examined the effector gene cluster 19A, which takes part in tumor formation. They deleted this cluster and some individual effectors and used microarray transcriptome analysis to discover several maize defense pathways associated with either the cluster or the individual effectors. Redkar et al. (2015), detected the function of the effector See1 using microarray analysis. They found that See1 modulates several processes during infection of maize, such as immune response, recovery of DNA synthesis, and plant cell division. See1 also plays a specific role in leaf tumor development. Villajuana-Bonequi et al. (2019), used RNA-Seq assays to reveal that DEGs related to the maize cell cycle, such as certain cyclins, are present in hyperplasic tumorous cells in which See1 promotes cell division. In the same way, Schurack et al. (2020), also used RNA-Seq analysis in their study on the effector UMAG_02297 to identify DEGs that respond specifically to the infection of wild-type or *UMAG_02297* knockout strains. *UMAG_002297*can slightly manipulate the gene expression of some of its targets, such as the auxin pathway in maize.

Colletotrichum graminicola effector protein 3 (CgEP3) is a small effector specific to *C. graminicola* with nucleo-cytoplasmic localization. It is a pre-penetration effector that plays a role in virulence during the early stages of infection of *C. graminicola* in maize (the biotrophic phase) and helps the fungus to penetrate the epidermis of the host plant (maize). The objective of this research is to study the maize genes that are induced or suppressed by the action of CgEP3 during the early stages of infection of maize with the fungus *C. graminicola* through RNA-Seq analysis.

RESULTS

Transcriptome analysis during the infection of maize leaves with *C. graminicola* at 24 hpi

We performed genome-wide transcriptional profiling with the objective of understanding the defense response of maize when infected with *C. graminicola*, particularly those pathways that could be affected by CgEP3. We used Illumina RNA-Seq analysis to compare maize leaves inoculated with the wild-type strain (WT) of *C. graminicola,* the Δ*CgEP3* null mutant, and negative control inoculated with water (mock) (Vargas et al., 2012). Plant tissue was harvested at 24 hpi, a time point at which the expression of *CgEP3* was maximum **(Figure 26)**. On the surface of leaves, mature and melanized appressoria were found similar to reports by Vargas et al. (2012) **(Figure 27)**. Three biological replicates, with an average of approximately 28 million reads, were performed **(Table 2)** Read libraries were examined using FastQC, which is a tool used for reads quality control (S. Andrews, 2010). The reads were aligned to a hybrid genome database composed of the *Zea mays* reference genome (B73_RefGen_v3) (Schnable et al., 2012) and the *C. graminicola* genome (strain M1.001_V1) (R. J. O'Connell et al., 2012) using Hisat2 (v.2.1.0) (Kim et al., 2019) with an alignment rate of approximately 84% **(Table 2)**.

Time	Library	Biological Replicate	Aligned exactly 1 time	Aligned >1 times	Aligned 0 times	Total reads	% Overall alignment rate
24 HPI	Mock	1	20,284,107	3,110,972	4,771,302	28,166,381	83,06%
		2	21,322,396	3,462,136	4,703,029	29,487,561	84,05%
		3	19,804,663	3,741,878	4,868,530	28,415,071	82,87%
	WT	1	20,863,401	2,480,091	4,410,631	27,754,123	84,11%
		2	24,754,938	2,995,186	4,754,843	32,504,967	85,37%
		3	23,369,547	2,740,391	5,324,013	31,433,951	83,06%
	Δ*CgEP3*	1	20,422,134	2,523,962	4,396,446	27,342,542	83,92%
		2	16,144,535	2,579,574	3,914,766	22,638,875	82,71%
		3	20,300,568	2,537,281	4,135,878	26,973,727	84,67%
Average			20,807,365	2,907,941	4,586,604	28,301,911	83,76%

Table 2. **Number of reads per treatment and biological replicate**. The number of reads that aligned once, more than once and zero times are shown. The overall percentage of alignment was approximately 84%.

We checked the level of correlation between the RNA-Seq libraries and the replicates of each treatment **(Figure 36)**. Pearson's correlation coefficient analysis showed a high correlation with each treatment: plants infected with wild-type strain (WT), mutant of CgEP3 strain (KO) and mock (M). The correlation coefficient was high: between 0.9 and 1 for WT and KO treatments and, between 0.6 and 0.7 for M treatment.

A principal component analysis (PCA) of RNA-Seq libraries was carried out to determine the variation between treatments and biological replicates **(Figure 37)** in RStudio, using R software and the DESeq2 package (Love et al., 2014; RStudio Team, 2015). The PCA revealed that there

was variation between treatments but not within the treatments. However, one biological replicate of the Δ*CgEP3* treatment group deviated from the other replicates, which could be due to differences in the progression of the infection in the maize leaves.

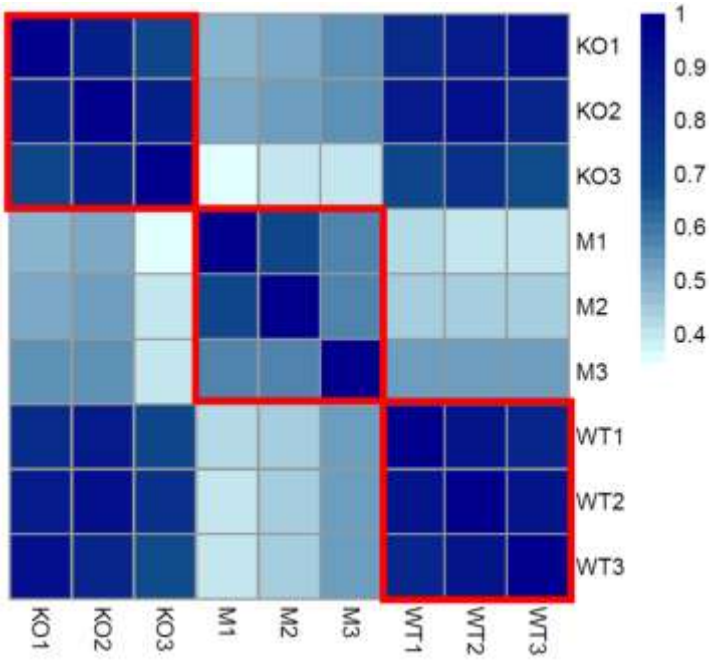


Figure 36. **Pearson's correlation heat map of RNA-Seq libraries** (DESeq2 v.1.28.1 and pheatmap v 1.0.12 R Packages (Kolde, 2012; Love et al., 2014; RStudio Team, 2015). Each small square represents the correlation between two pairs of libraries. Red squares indicate the correlation between replicates of each treatment: plants infected with wild-type strain (WT), mutant of *CgEP3* strain (KO) and mock (M). The correlation coefficient was high: between 0.9 and 1 for WT and KO treatments and, between 0.6 and 0.7 for M treatment.

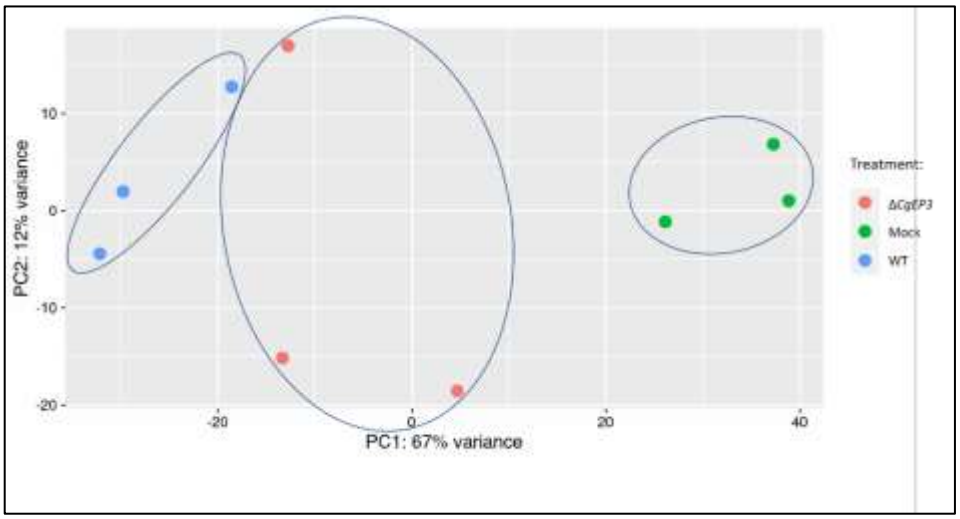


Figure 37. **Principal Components Analysis (PCA) of RNA-Seq** (DESeq2 v.1.28.1R and plotPCA R Packages (Love et al., 2014; RStudio Team, 2015). Colored dots represent individual biological replicate during the infection at 24hpi. These were grouped by similarity of treatment (circles).

After alignment with Hisat2, the abundance of reads was calculated, and gene expression levels were estimated. Differential expression analysis between pairwise comparisons and statistical

analyses was conducted using DESeq2 R package (v.1.28.1) (Love et al., 2014) using R and RStudio (RStudio Team, 2015). DEGs were considered those for which log_2foldchange was ≥2 or ≤-2, and $p_{adjusted}$ was <0.05. A summarized flowchart of this process is shown in **Figure 38**. Gene functions were annotated using public databases and manually. BLAST-Koala Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2016), Gene Ontology (GO) (Carbon et al., 2019; Juanjuan Chen et al., 2006), InterPro (Jones et al., 2014; Mitchell et al., 2019), PfamScan (Mistry et al., 2007), Phytozome 10 (Goodstein et al., 2012), Panther (Mi et al., 2013; P. D. Thomas et al., 2003), KOG (Bioinformatics et al., 2003) and MaizeGDB (Portwood et al., 2019) (**Supplementary Tables 7 and 9**).

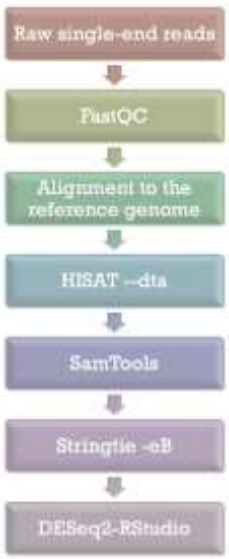


Figure 38. **Summarized flowchart of the RNA-Seq analysis process**. The quality of sequenced libraries was analyzed with FastQC High Throughput Sequence QC Report (v.0.11.7). The reads were mapped to the *Zea mays* (B73 RefGen_v3) and *Colletotrichum graminicola* (C. *graminicola* M1.001 v1) hybrid genome databases using Hisat2 (v.2.1.0). For each library, the number of reads mapped to each gene was counted with StringTie -eB (v1.3.5). Differential expression analysis between several pairwise comparisons and statistical analysis were performed with the DESeq2 (v.1.28.1) R package. Further details are added in the materials and methods section.

A comparison of plants inoculated with the WT strains against the mock-inoculated plants identified 1,916 DEGs **(Figure 39A)**. These genes, which are known as disease response genes, are regulated in the presence of the pathogen. To study the role of CgEP3 in the disease process, DEGs identified from Δ*CgEP3*-inoculated maize leaves were compared with those from WT-inoculated leaves. These DEGs are regulated in the absence of the effector and are referred to as effector-responsive genes. Among these, there are a total of 56 DEGs, 35 of which are upregulated and 21 are downregulated **(Figure 39A and Supplementary Table 7)**. This indicates that there are 35 and 21 DEGs that could, respectively, be suppressed or induced by the action of CgEP3, when maize was infected with the wild-type strain. These were selected for further analyses since, in both cases, it is unknown whether the action of CgEP3 in these genes is affected directly or indirectly. Finally, the comparison KO_Mock represents another disease-responsive genes related to *CgEP3* gene deletion (**Figure 39A**). However, this study is concentrated mainly on the CgEP3 effector-responsive genes.

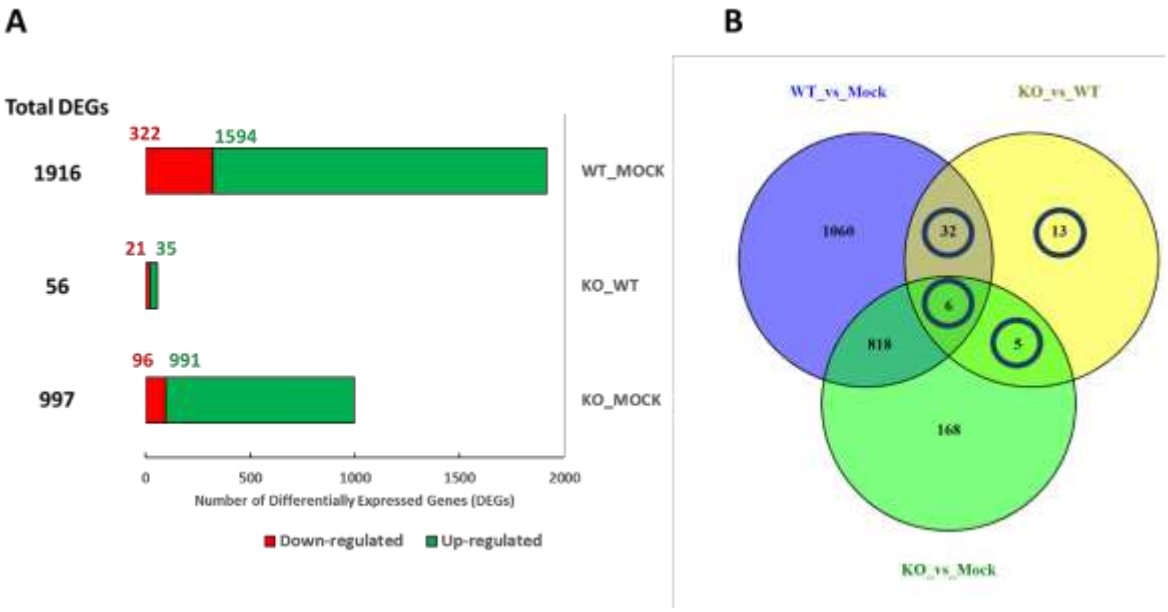


Figure 39. **Differentially expressed genes (DEGs) in RNA-Seq experiment and comparisons between treatments. A)** DEGs found after the comparison of the treatments. WT_Mock represents maize leaves inoculated with WT strains compared with plants inoculated with the Mock. These are disease-responsive genes. There are 1,916 DEGs, out of which 322 are upregulated and 1,594 are downregulated. KO_WT represents maize leaves inoculated with Δ*CgEP3* compared with those inoculated with WT strains. These are effector-responsive genes. There are 56 DEGs, out of which 35 are upregulated (DEGs that could be suppressed in presence of CgEP3 effector in wild-type inoculated plants) and 21 DEGs that are downregulated (genes that could be induced in presence of CgEP3 effector in wild-type inoculated plants). **B)** Venn diagram plot (Oliveros, 2015): it represents shared DEGs between comparisons. Blue circles represent 56 DEGs that respond to the CgEP3 effector.

Figure 39B illustrates the relationships between the comparisons of treatments and shared DEGs through a Venn diagram. Of the 56 DEGs responsive to CgEP3, 32 DEGs responded to both the disease and the effector, while 13 DEGs responded only to the effector. Five of the DEGs were responsive to other diseases related to *CgEP3* gene deletion, and six of the DEGs responded to all the comparisons.

56 significant DEGs were obtained after statistical analysis using DESeq2 (v.1.28.1) (Love et al., 2014). These are reflected in the volcano plot **(Figure 40A)**. This indicates that there are a few genes that respond to the effector. The heatmap shows gene expression through $\log_2$foldchange in the different comparisons **(Figure 40B)**. When comparing KO vs. WT with WT vs. Mock, there was a group of DEGs that was upregulated in KO vs. WT but downregulated in WT vs. Mock. These DEGs were suppressed by CgEP3 effector. In the same way, there was a group of DEGs that were downregulated in KO vs. WT, but upregulated in KO vs. WT. These DEGs were induced by CgEP3.

As a preliminary approach towards categorization of the DEGS, we employed GO terms using the agriGO website (Du et al., 2010a; T. Tian et al., 2017) to examine the functional categories of each DEG. In case of both upregulated and downregulated DEGs the most frequently found functional categories were the common GO terms, such as “metabolic process,” “catalytic activity,” “binding” and “cellular process” **(Figure 41)**.

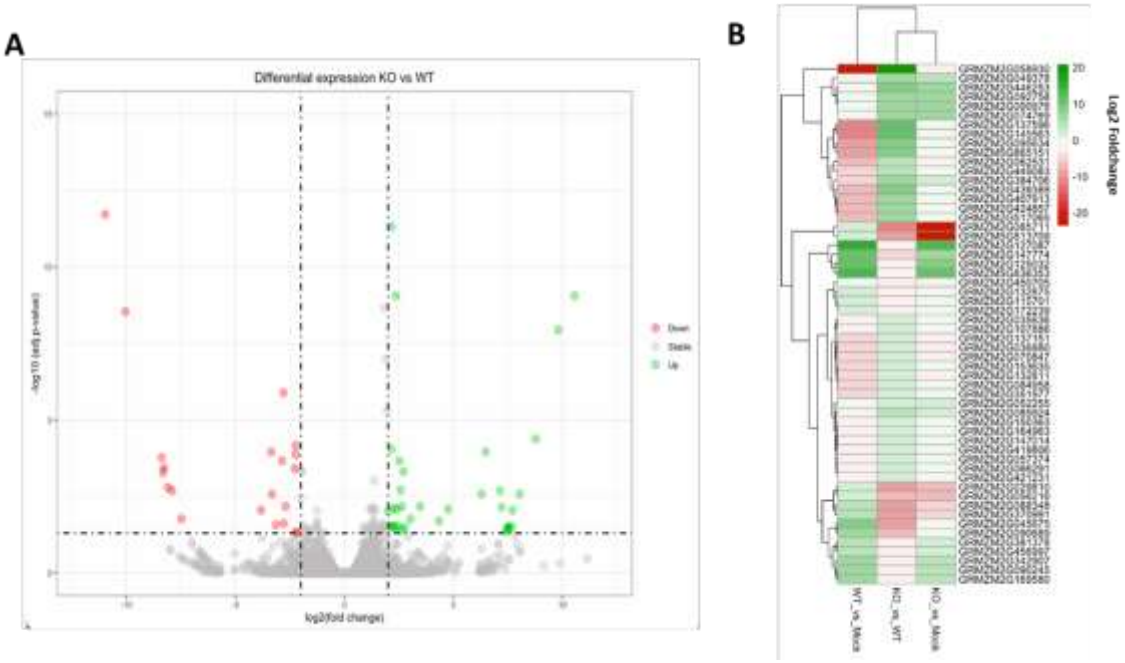


Figure 40. Volcano and heat map plots: A) Volcano plot (R package ggplot2 3.3.2 from RStudio Team, 2015; Wickham, 2016) represents 56 significant DEGs (designated when log_2foldchange is ≥2 or ≤-2, and $p_{adjusted}$ is <0.05). **B)** Heatmap (R package pheatmap 1.0.12 from Kolde, 2012; RStudio Team, 2015) plot shows gene expression levels through log_2foldchange in the different comparisons. DEGs that are upregulated in KO vs. WT compared to WT vs. Mock are suppressed by CgEP3 effector. DEGs that are downregulated in KO vs. WT compared with WT vs. Mock are induced by CgEP3 effector.

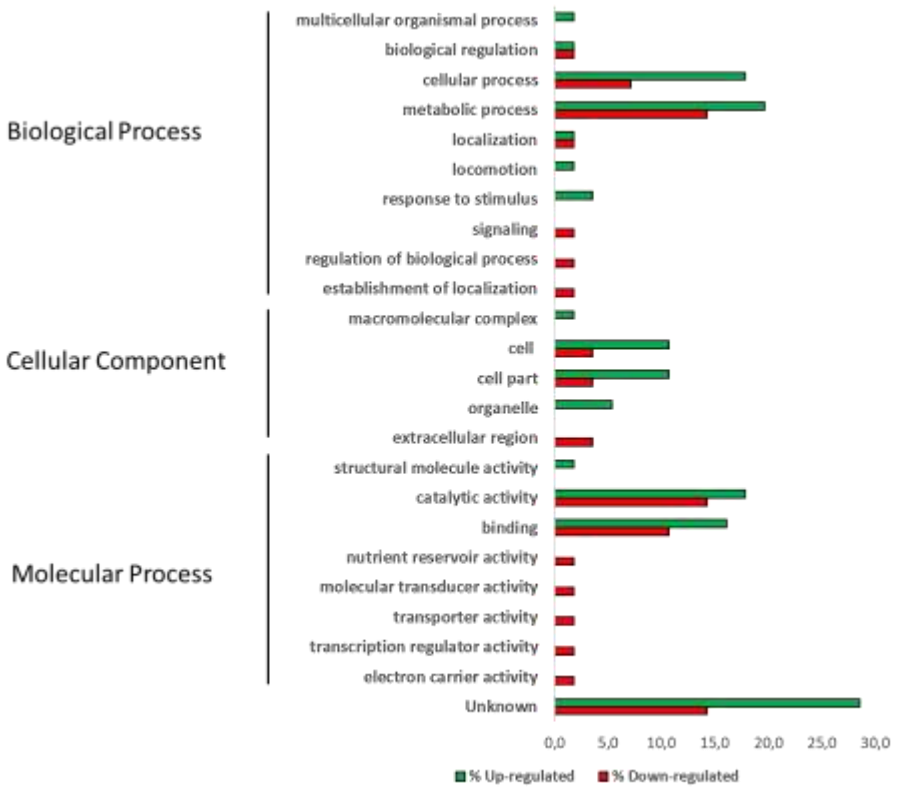


Figure 41. Gene Ontology Terms identified using agriGo (Du et al., 2010; Tian et al., 2017). The GO terms of 56 DEGs that respond to CgEP3 are shown. The most frequent functional categories in cases of both upregulated and downregulated DEGs were "metabolic process", "catalytic activity", "binding" and "cellular process".

GO enrichment analysis was carried out to identify significantly enriched GO terms using agriGO (Du et al., 2010a; T. Tian et al., 2017). The upregulated genes belonged to the categories "chlorophyll metabolic process," "terpenoid biosynthetic process," "cellular lipid metabolic process," "pigment biosynthetic process," "1-deoxy-D-xylulose-5-phophate synthase," "protochlorophyllide reductase activity," "ligase activity", "forming nitrogen-metal bonds" **(Supplementary Table 10A and Supplementary Figure 4)**. This suggests that CgEP3 could directly or indirectly affect the chlorophyll biosynthesis pathway, lipid metabolism pathway, or even secondary metabolism through the terpenoid biosynthesis pathway.

In the same manner, the downregulated genes belonged to the categories "sulfate assimilation," "sulfate transport," "sulfate transmembrane transporter activity," "sulfate adenylyltransferase (ATP) activity," "monooxygenase activity," etc. **(Supplementary Table 10B and Supplementary Figure 5)**. This suggests that CgEP3 is detected by plants, and it can activate defense responses, in which sulfur metabolism can play an important role (Capaldi et al., 2015; X. Fu et al., 2016; Rausch & Wachter, 2005).

To further understand the functions of the DEGs and to assign them to biological pathways, we consulted public databases and literature to classify them into putative functional categories that are shown in the following table **(Supplementary Table 7)**.

To determine the subcellular localization of proteins that can be affected by CgEP3, we performed *in silico* analysis with different applications **(Supplementary Table 6)**. Most of these predicted locations matched those of experimentally proven results. The proteins were predicted to be present in the nucleus (GRMZM2G049378, GRMZM2G107886, GRMZM2G169580, GRMZM2G381378, GRMZM2G115701, GRMZM2G066291, GRMZM5G865151, GRMZM2G449083, GRMZM2G090689, GRMZM2G147014, and GRMZM2G153635); chloroplast (GRMZM2G150363, GRMZM2G084958, GRMZM2G351977, GRMZM2G137151, GRMZM2G070847, GRMZM2G419806, GRMZM2G036880, GRMZM2G450705, GRMZM2G127087, GRMZM2G421231, GRMZM2G132811, GRMZM2G057374, GRMZM5G836353, GRMZM2G092758, and GRMZM2G164963); or cytoplasm (GRMZM2G446253 and GRMZM2G085924) or were predicted to be secreted proteins (GRMZM2G456997, GRMZM2G125032, GRMZM2G085711, GRMZM2G090245, and GRMZM5G813709). However, the remaining proteins were rather difficult to predict and returned ambiguous locations. Overall, we observed that CgEP3 could have nuclear and cytoplasmic subcellular localization based on the heterologous expression of *CgEP3* in *N. benthamiana* **(Chapter I, Figure 27)**. We found numerous genes related to the nucleus and other compartments, which may constitute downstream responses to CgEP3 action.

DIFFERENTIALLY EXPRESSED GENES AFFECTED BY CgEP3

GENES RELATED TO DEFENSE RESPONSE

Two genes were induced by CgEP3 – *GRMZM2G456997*, and *GRMZM2G125032* **(Supplementary Table 7 and Figure 42)**. Both genes encode pathogenesis-related proteins (PRs), which are important during the PTI defense response (De Jesus Miranda et al., 2017). These proteins are inducible and play a role against pathogen infection and can also act as indicators of systemic defense (De Jesus Miranda et al., 2017; Sels et al., 2008).

The maize gene *GRMZM2G456997* was annotated as a pathogenesis-related protein PRB1-3-like (Bhat et al., 2019a) **(Supplementary Table 7 and Figure 42)**. Bhat et al., (2019) analyzed *the C. graminicola*-maize pathosystem and specifically searched for pathways related to defense response in maize. This protein has lysM domains in its structure that can be linked with chitin elicitor binding protein (Bhat et al., 2019a). Shi et al. (2018), found *GRMZM2G456997* to be upregulated against the fungus *Setosphaeria turcica*, a hemibiotrophic pathogen that causes northern corn leaf blight disease (NCLB). Thus, this gene can act in maize defense against NCLB disease infection. "The Chinese patent by Chu and Ding (2015) (CN104498507A)" reported *GRMZM2G456997* to be constitutively expressed in maize plants, and thereby provide resistance to maize against corn sheath blight caused by the fungus *Rhizoctonia solani.* Hence, this gene plays an important role in plant defense.

GRMZM2G125032 encodes a β-1,3-glucanase (ZmGns), which is a member of the pathogenesis-related protein 2 (PR-2) family that participates in response to biotic and abiotic stresses (Xie et al., 2014) **(Supplementary Table 7 and Figure 42)**. It is usually associated with plant defense and enhances host resistance to various pathogens. *ZmGns* is expressed during bacterial infection (*Clavibacter michiganensis* subsp. *nebraskensis, Erwinia chrysanthemi, Erwinia stewartia*), as well as fungal infection (*Aspergillus flavus*). When *ZmGns* is expressed constitutively in *Arabidopsis thaliana,* it shows antibacterial and fungal activity against the pathogens *Pseudomonas syringae* pv. *tomato* DC3000 and *Botrytis cinerea,* respectively (Xie et al., 2014). Vargas et al. (2012) showed that this gene is induced against *C. graminicola* infection in maize leaves at 72 hpi, and later, Christie et al. (2017), it is induced against *Cercospora zeina* infection causing gray leaf spot in maize.

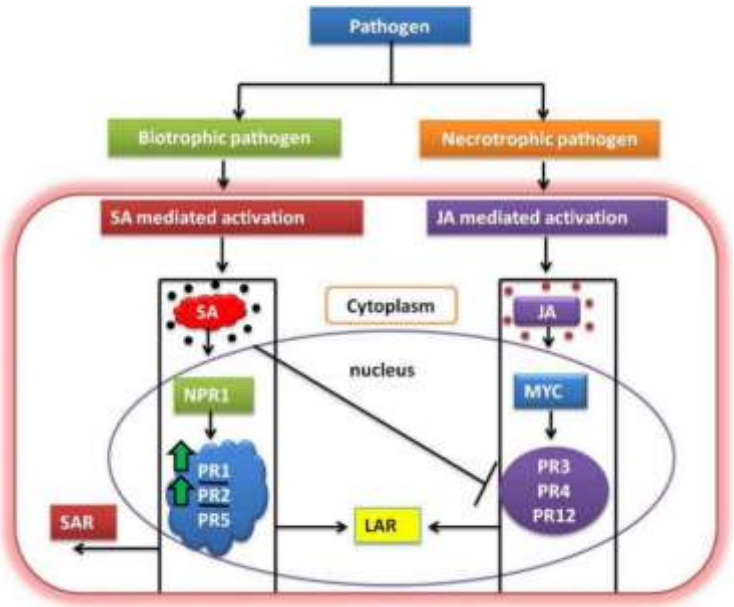


Figure 42. CgEP3 effector induces DEGs related to plant defense response. An overview of activation of signaling cascades in plants after biotrophic and necrotrophic pathogenic infection. Green arrows with black borders represent the genes that are induced by CgEP3: PRB1-3-like and PR2. Because these genes are activated by salicylic acid (SA) accumulation (S. Ali et al., 2018), CgEP3 can possibly induce SA signaling pathway. This figure has been modified from the original that is found in the article by Ali et al., 2018.

GENES RELATED TO PLANT HORMONES

Plant hormones are important molecules that fulfill different functions and play either an antagonistic or a synergistic role during the entire life of the plants. They are small and act as signaling molecules (Abdelrahman et al., 2019; Abdelrahman & Jogaiah, 2020; De Bruyne et al., 2014; De Zelicourt et al., 2013; Grover et al., 2013). They monitor plant growth and development processes, as well as the transmission of environmental cues and defense responses against biotic and abiotic stresses (Abdelrahman & Jogaiah, 2020). CgEP3 can suppress several genes involved in plant hormone signaling, such as *GRMZM2G150363* (related to abscisic acid (ABA) biosynthesis), and induce several others, such as *GRMZM2G370991* (related to ethylene signaling) and *GRMZM2G147774* (related to gibberellins (GA) biosynthesis) **(Supplementary Table 7, Figures 43, 44, and 45)**.

Related to ABA biosynthesis

The maize genes *NCED8 and GRMZM2G150363* encode a 9-cis carotenoid cleavage dioxygenase (NCED) 8 protein (Dutta et al., 2019a) **(Figures 43, 47, and Supplementary Table 7)**. NCED participates in the first step of ABA biosynthesis, excising the double bond between the 11th and 12th positions of 9-cis neoxanthin or 9-cis violaxanthin molecules (Dutta et al., 2019a; Schwartz et al., 1997). NCED genes play a vital role in the plant kingdom. Together with carotenoid cleavage dioxygenase enzymes, they mediate the production of apocarotenoids through oxidative damage of the carotenoids (Dutta et al., 2019a; X. Hou et al., 2016). There are important molecules derived from carotenoids that play a role in plant physiology, such as ABA, strigolactone, and volatile compounds (Vallabhaneni et al., 2010). Ding et al. (2019), found that this gene is downregulated in maize infected with the hemibiotrophic fungus *Cochliobolus heterostrophus* at 24 hpi. Homologs of this gene in rice (*LOC_Os02g47510.1*) is downregulated against brown planthopper pest infestation caused by *Nilaparvata lugens* (F. Wang et al., 2015). Jung et al. (2014) showed that this gene enhances resistance in rice against *Xanthomonas oryzae* pv. *oryzae.*

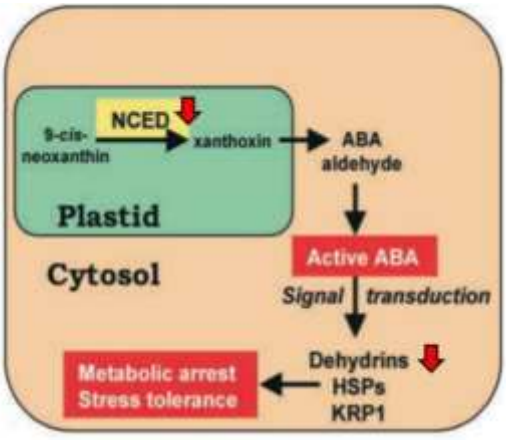


Figure 43. CgEP3 effector may modulate ABA response. Scheme showing the synthesis of ABA via chloroplastic NCED followed by the downstream signaling. CgEP3 may suppress NCED and the Dehydrin proteins. This figure has been modified from the original that is found in the article by Pastori et al., 2003.

Related to ethylene signaling

In maize, *GRMZM2G370991* encodes an ethylene-insensitive 2-Like protein (EIN2-Like) (Mei et al., 2019) **(Figure 44 and Supplementary Table 7)**. Homologs of this gene in *A. thaliana*

(*AT5G03280.1*) encodes *ethylene-insensitive 2* (*AtEIN2*). It is a positive regulator of ethylene responses. EIN2 is found in the endoplasmic reticulum and participates in the ethylene signaling pathway. It is associated with ethylene receptors (Bisson et al., 2009; Bisson & Groth, 2010; Ju et al., 2012). There are some nuclear transcription factors, such as EIN3 and ERF1, which participate downstream of EIN2 and acts as transcriptional regulators in response to ethylene (An et al., 2010; Ju et al., 2012; Solano et al., 1998). Zhang et al. (2020), reported that their role in resistance to biotrophic and hemi-biotrophic pathogens was ambiguous (Al-Daoud & Cameron, 2011; H. Chen et al., 2009; Cyril Zipfel, 2013) and non-existent against necrotrophic pathogens (Trusov et al., 2009). Homologs of this gene in rice (*Os07g06130.1*) encodes the protein OsEIN2.1, which is downregulated during *Magnaporthe oryzae* infection in rice plants (cv TN67). It occurs in different stages: 24–48 hpi in the biotrophic phase and 72 hpi in the necrotrophic phase. However, it is still unknown how the regulatory mechanisms are related among the members of EIN2 family (Salvador-Guirao et al., 2018).

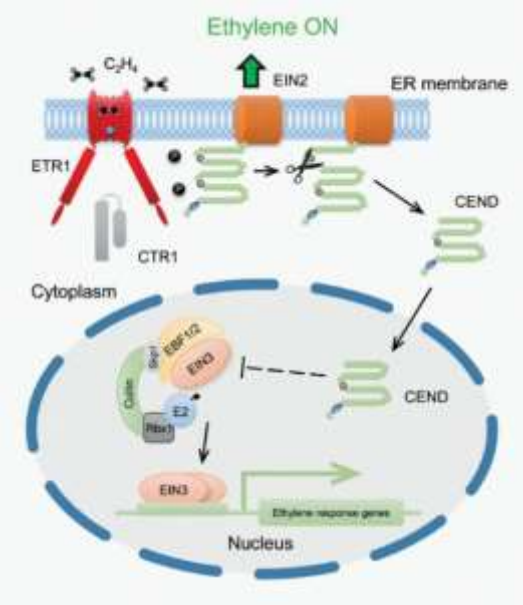


Figure 44. CgEP3 effector may induce ethylene signaling pathway. A hypothetical model of EIN2 in the ethylene signaling pathway. CgEP3 can induce EIN2 and in turn, activate downstream signaling. Green arrow with black border represents the gene that can be induced by CgEP3. This figure has been modified from the original that is found in the article by Ji & Guo, 2013.

Related to gibberellin biosynthesis

The maize gene *GRMZM2G147774* is annotated as cytochrome P450, E-class, group I (Jones et al., 2014; Mitchell et al., 2019) **(Figures 45, 47, and Supplementary Table 7)**. Homologs of this gene in *A. thaliana* (*AT3G14690.1*), which encodes a cytochrome P450 (CYP72A15), participates in the biosynthesis pathway of gibberellins. Overall, this hormone plays an important role in controlling different processes related to the development and growth of plants. CYP72A15 converts GA9 and GA12 into GA20 (and an unknown product) and GA53 (Juan He et al., 2019). Sham et al. (2017) reported this gene was induced in response to *B. cinerea* infection at 24 hpi. In the work of Ohta & Mizutani (2004), this gene was induced in systemic leaves when plants were infected with *P. syringae* pv. *tomato* DC3000 avirulent strain [Pst(*avr-Rtp2*)].

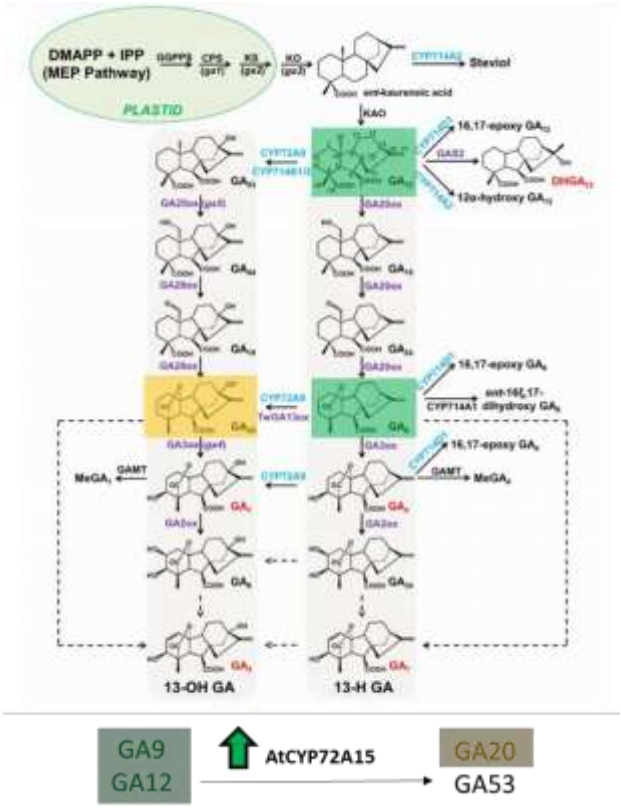


Figure 45. CgEP3 induces the gibberellin biosynthesis pathway. Simplified gibberellic acid (GA) metabolic pathway in flowering plants. CgEP3 can induce Cytochrome P450 (AtCYP72A15) which acts positively in GA biosynthesis pathway. Green arrow with black border represents the gene that can be induced by CgEP3. This figure has been modified from the original that is found in the article by He et al., 2020.

TRANSCRIPTION FACTORS

Transcription factors (TFs) are the master controllers of different vital processes related to plant growth and development. They participate in plant responses to external cues, such as biotic and abiotic stresses, and can regulate plant defense responses. Due to their important roles, TFs are subjected to different controls at either transcriptional or post-transcriptional levels, as well as post-transcriptional modifications (Ng et al., 2018). We found CgEP3 to suppress several TFs, including *GRMZM2G049378* (MYB) and *GRMZM2G107886* (CO-Like/BBX) and induce other TFs, including *GRMZM2G169580* (GT, trihelix), *GRMZM2G381378* (WRKY), and *GRMZM2G115701* (TPL-Tubby) **(Supplementary Table 46 and Figure 7)**.

The maize gene annotated as *ZmMYBR115* (*GRMZM2G049378*), encodes an MYB transcription factor (Gray et al., 2009) **(Figure 46 and Supplementary Table 7)**. The family of MYB proteins has wide functional diversity and can be found in all eukaryotes. The majority acts as TFs that with a variable number of MYB domains that allow linkage to DNA. MYB TFs participate in the regulation of networks and processes implicated in metabolism, development, and biotic and abiotic stresses (Dubos et al., 2010; Y. Zheng et al., 2016). Homologs of this gene in *A. thaliana* (*AT1G72250.1*) encode a myb-related PPR2 protein that plays a role in the resistance of *Peronospora parasitica* (Federspiel et al., 2010).

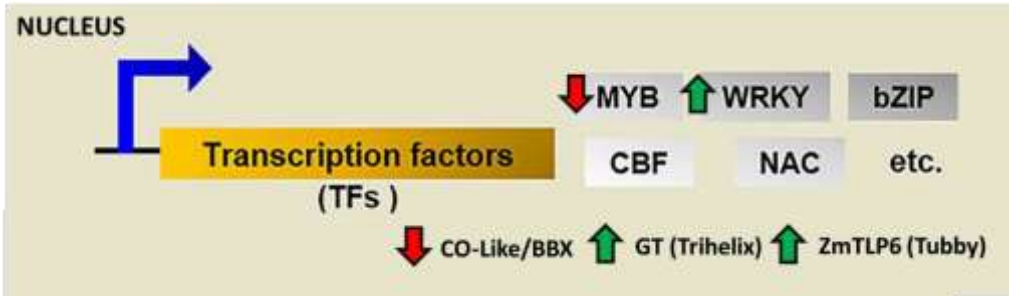


Figure 46. CgEP3 can modulate plant transcription factors (TFs). Different kinds of TFs can induce stress-responsive genes for plant tolerance and resistance. CgEP3 could suppress MYB and CO-Like/BBX TFs and induce WRKY, GT (Trihelix) and ZmTPL6 (Tubby) TFs. Red and green arrows with black borders represent genes that are suppressed or induced respectively by CgEP3. This figure has been modified from the original that is found in the article by Chi et al., 2019. Suppressed.

In maize, the gene *GRMZM2G107886* is annotated as *ZmCOL16* (CONSTANS-LIKE 16) and controls blooming in conjunction with photoperiods and also responds to abiotic stresses (N. Song et al., 2018) **(Figure 46 and Supplementary Table 7)**. All members of the COL protein family have two domains that are conserved in their structure: a zinc finger region named B-box (BBX) involved in protein-protein interaction (Borden, 1998; Wenkel et al., 2006), and the CTT domain (CO, CO-Like), which is implicated in the nuclear localization of the proteins (Wenkel et al., 2006). BBX proteins also play important roles in response to biotic stress (Shalmani et al., 2019). Ding et al. (2019), found that this gene is downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. In the work of Barah et al. (2015), homologs of this gene in *A. thaliana* (*AT1G25440.1*) was regulated against several stresses, including cold, salt, heat, and a combination of cold and plants inoculated with flg22 peptide. flg22 is a bacterial elicitor that reproduces biotic stress. It is recognized by plants and triggers an immune response (Barah et al., 2015; Jelenska et al., 2017).

In maize, *GRMZM2G169580* is predicted to encode a family of triple-helical transcription factors (P. Li et al., 2010) **(Figure 46 and Supplementary Table 7)**. This family of TFs is also known as the GT family, and the proteins are composed of three helices that are repeated in tandem (helix-loop-helix-loop-helix). GT TFs are frequently found in plants (Wang et al., 2019). They can be found in different species, such as *A. thaliana* (with 30 *GT* genes) (Kaplan-Levy et al., 2012) or rice (with 31 *GT* genes) (Ji et al., 2015). The GT TF family is classified into five different clusters: GT-1, GT-2, SIP1, GTγ, and SH4 (Kaplan-Levy et al., 2012). GT genes act in plant growth and development against diverse ambient stresses and are expressed strongly in plant leaves (Wang et al., 2019). Following the phylogenetic tree of GT proteins by Wang et al. (2019), we included *GRMZM2G169580* in the GTγ class. Homologs of this gene in rice, *LOC_Os02g43300.1* is also classified into the GTγ class. An example of GT-γ genes in rice is *OsGTγ-1,* which is important during salt stress since it enhances salinity tolerance (Y. Fang et al., 2010). In contrast, GTγ can also play a role against biotic stresses, such as *TuGTγ-3,* which imparts resistance to *Triticum urartu* against the stripe rust caused by *Puccinia striiformis* (Ding et al., 2016).

In maize, *GRMZM2G381378* was annotated as ZmWRKY83, following the study of Wei et al. (2012) (**Figure 46 and Supplementary Table 7)**. The family of WRKY TFs is important during biotic stresses, such as fungal, bacterial, nematodal, and viral infections, as well as against insect infestations. ZmWRKY83 belongs to Group III of WRKY transcription factors that are associated with the defense response. Proteins of this group have a variable motif of C2HC-type zinc finger-like domains at the C-terminal region of the WRKY domain (K. Wei et al., 2012). Homologs of this

gene in rice, *LOC_Os07g48260.1*, encodes a WRKY47 (Ross et al., 2007) that combats biotic and abiotic stresses. *OsWRKY47* was upregulated against the rice blast fungus *M. oryzae* during the early defense response at 24 hpi. OsWRKY47 enhances resistance, indicating its positive role in rice defense against this fungus (Wei et al., 2013). Homologs of the maize gene in *A. thaliana* (*AT3G56400.1*) encodes a WRKY46 protein that is clustered in Group III of WRKY TFs, because it has a WRKY domain and C2HC zinc-finger motif (Eulgem et al., 2000; Yanru Hu et al., 2012). Thus, *AtWRKY46* is expressed during infection by both *P. syringae* and salicylic acid (Yanru Hu et al., 2012).

The maize *GRMZM2G115701* gene is predicted to encode the ZmTPL6 transcription factor (Yulong et al., 2016) (**Figure 46 and Supplementary Table 7)**. TUBBY-like or TPL are found in eukaryotes (C. P. Lai et al., 2004; Q. Liu, 2008) and can act as transcription factors (Boggon et al., 1999). In general, the structure of these proteins in the C-terminal region has a TPL domain (approximately 270 amino acids in length), which often contains an F-box domain that is preserved (Gagne et al., 2002; Zefeng Yang et al., 2008). Proteins that have TPL and F-box domains at their C-terminal region are often known to conserve other variable domains which play a role in protein-protein interactions, e.g., leu-rich repeat, tetratricopeptide repeat, WD40 repeat, and kelch repeat (Jain et al., 2007). TPLs participate when plants are subjected to abiotic stress. Additionally, *ZmTPL6* is upregulated in response to ABA (Yulong et al., 2016). In a study by Villajuana-Bonequi et al. (2019), this gene (whose encoded protein is denominated ZmFBX154.1) is activated during *Ustilago maydis* infection. It is expressed in hyperplastic tumor cells, although the function of the majority of F-box genes in plants remains unclear. Homolog of this gene in rice (*LOC_Os02g47640.1*) encodes the OsTPL8 protein. *OsTPL8* is induced in the bacterial infection of *Xanthomonas oryzae* pv. *oryzae,* which causes bacterial blight at 24 hpi (Kou et al., 2009). Wang et al. (2019), in their study related homologs of this gene in *A. thaliana* (*AT2G18280.1*) to the transcription of genes that fulfill a function in the cell wall, such as biosynthesis of homogalacturonans. Another study related the gene to the formation of galls, which are structures on plants bodies produced by some pathogens, such as fungi (Takeda et al., 2019).

GENES RELATED TO PHOTOSYNTHESIS

Photosynthesis is one of the most important processes that maintains life on the planet. Plant photosynthesis is based on two different reactions that occur in separate chloroplast compartments. Light reaction occurs in the membranes of thylakoids. In this step, water is excised into oxygen (released), protons and electrons, which, in turn, are utilized to generate adenosine triphosphate (ATP) and nicotinamide-adenine dinucleotide phosphate (NADPH). Both are considered as molecules that store energy. Subsequently, the dark reaction or Calvin cycle occurs in the stroma of chloroplasts. Here, both ATP and NADPH are used for carbohydrate biosynthesis from CO_2 (M. P. Johnson, 2016). We found several genes related to the photosynthetic process that could be affected by CgEP3. It suppressed *GRMZM2G084958*, *GRMZM2G419806*, and *GRMZM2G137151* (related to chlorophyll and carotenoid biosynthesis and other compounds), *GRMZM2G351977*, *GRMZM2G036880*, *GRMZM2G070847*, and induced *GRMZM2G450705* (related to the photosystems and electron transfer chain) (**Supplementary Table 7, Figures 47 and 48**).

Related to chlorophyll and carotenoid biosynthesis and other compounds

The maize gene *GRMZM2G137151* encodes the 1-deoxy-D-xylulose-5-phosphate synthase 1 protein (DXS1) (Zhang et al., 2019) (**Figure 47 and Supplementary Table 7**). The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is of vital importance to plants. This pathway, localized in the chloroplasts, plays a role in the biosynthesis of plant isoprenoid precursors, such as isopentenyl diphosphates and dimethylallyl diphosphates (Cordoba et al., 2011; Lili Zhang et al., 2019). Isoprenoids have diverse functions in plants, such as growth and development control, primary metabolism, biosynthesis of cell walls, and intracellular signaling, as well as against biotic and abiotic stresses (de Luna-Valdez et al., 2020; Eisenreich et al., 2001; Lichtenthaler, 1999; Sacchettini & Poulter, 1997). This varied cluster of molecules contain important components, such as chlorophylls, carotenoids, tocopherols, hormones (such as GAs, ABA, and strigolactones), and plastoquinones, among others (Bouvier et al., 2005; de Luna-Valdez et al., 2020; Umehara et al., 2008; Lili Zhang et al., 2019). The MEP pathway has been characterized in *A. thaliana* and is considered as a model for plant systems. The first step of this pathway is the conversion of glyceraldehyde 3-P and pyruvate into 1-deoxy-D-xylulose-5-phosphate. This reaction is catalyzed by the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Cordoba et al., 2011; Lili Zhang et al., 2019). Several studies have shown that this enzyme controls one of the main reaction steps of bacterial and plant MEP pathways (de Luna-Valdez et al., 2020; Estévez et al., 2001; Muñoz-Bertomeu et al., 2006; Wright et al., 2014). Plant isoprenoids accumulate when the concentration of DXS1 increases, and vice versa. Thus, depending on DXS1 abundance, the amounts of different components, such as carotenoids, chlorophylls, hormones (ABA and GAs), and monoterpenes increase or decrease (de Luna-Valdez et al., 2020; Estévez et al., 2001; T. Zhang et al., 2018).

Ding et al. (2019) reported this gene to be related to pathogenesis and to be downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. Kim et al. (2017) selected putative resistance transcripts for resistance to oomycete downy mildew (DM) infection (caused by several species of *Peronosclerospora* and *Sclerophthora genus, such as P. sorghi, P. maydis, and S. macrospora)*. Both transcripts produced by the *GRMZM2G137151* gene are upregulated against DM infection in CML228 maize resistant plants. Homologs of this gene in rice (*LOC_Os05g33840.1*), in the study by Kumari et al. (2017), is upregulated against *R. solani* infection. In the work of Venu et al. (2010), *LOC_Os05g33840.1* is downregulated in beet armyworm (*Spodoptera exigua*) pest infestation in rice. This is related to the secondary metabolism pathway and hemiterpenes. Homologs of this gene in *A. thaliana* (*AT4G15560.1*) induced plant resistance against the herbivore *Spodoptera littoralis* infestation (Mitra et al., 2021). The rate of the MEP pathway is controlled by DXS, which is important for producing metabolites that are utilized during photosynthesis. During *S. litloralis* infestation, the increase in ROS levels allows β-carotene transformation to β-cyclocitral, which can directly inhibit the action of DXS enzyme (Mitra et al., 2021). Agudelo-Romero et al. (2008) reported this gene to be downregulated against *tobacco etch potyvirus* infection in *A. thaliana.* This enzyme is important for the development of chloroplasts in *A. thaliana* (Mandel et al., 1996).

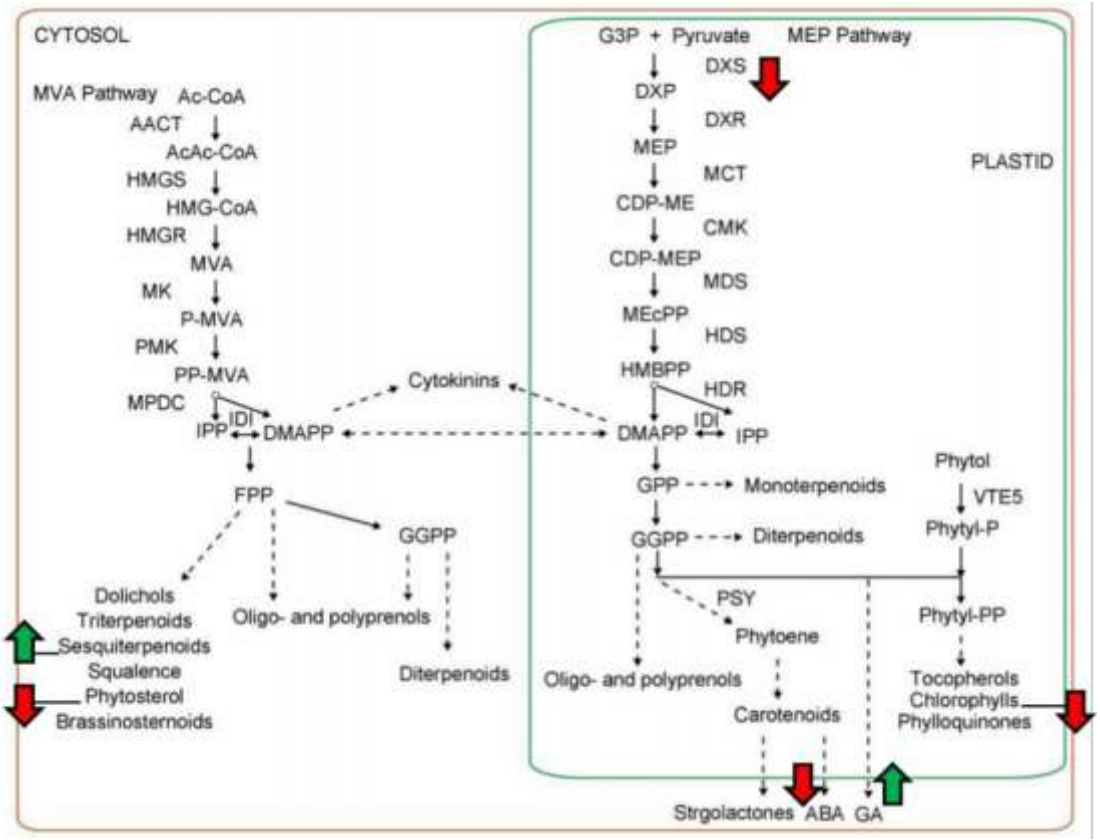


Figure 47. CgEP3 effector can modulate MEP pathway through suppression of DXS enzyme. Abbreviated scheme of plant mevalonate (MVA) and MEP pathways and the products that are biosynthesized downstream. CgEP3 could suppress DXS enzyme which controls one of the main reaction steps of MEP pathway. Then CgEP3 could modulate plant defense response through downstream products of MEP pathway. Other pathways that could be affected by CgEP3 are chlorophyll and phytosterol biosynthesis (suppressed), and sesquiterpenoid biosynthesis (induced). Red and green arrows with black borders represent genes or pathways that are suppressed or induced respectively by CgEP3. This figure has been modified from the original that is found in the article by Zhang et al. (2019).

The maize gene *GRMZM2G419806* encodes magnesium protoporphyrin IX chelatase protein (CHLD) (Zhang et al., 2019) **(Figure 48 and Supplementary Table 7**). Chlorophylls are tetrapyrrole molecules that are indispensable for plant photosynthesis. The first step of biosynthesis corresponds to the generation of 5-aminolevulinic acid (ALA), which is the precursor of tetrapyrrole molecules such as chlorophylls and protohemes (Beale, 1999; Masuda & Fujita, 2008; Tanaka & Tanaka, 2007; Jinyao Zhang et al., 2019). The next step was the synthesis of protoporphyrin IX. From this molecule, a Mg^{2+} cation is included to form Mg-protoporphyrin IX, a reaction that is catalyzed by the magnesium chelatase enzyme (ChL), which contains three subunits: ChLH, ChLI, and ChLD (Masuda & Fujita, 2008; R. Tanaka & Tanaka, 2007; Jinyao Zhang et al., 2019). Finally, there are some downstream steps in the synthesis of chlorophyll molecules (Masuda & Fujita, 2008; R. Tanaka et al., 2011; Tanaka & Tanaka, 2007; Jinyao Zhang et al., 2019), including the enzyme protochlorophyllide reductase A (ZmPORA), which forms chlorophyllide a (Kretschmer et al., 2017; Jinyao Zhang et al., 2019). Two studies have shown that this gene is downregulated againstin *C. Zeina* (Christie et al., 2017) and the hemibiotrophic fungus *C. heterostrophus* at 24 hpi (Ding et al., 2019). The thesis dissertation of Terpstra, (2012) reported that homologs of this gene in *A. thaliana* (*AT5G45930.1*) takes part in QTL: QDP1, which enhances disease symptoms and susceptibility to *P. syringae* pv. *tomato.*

The maize gene *GRMZM2G084958* encodes a protochlorophyllide reductase A (ZmPORA) that participates in chlorophyll biosynthesis (Kretschmer et al., 2017; Zhan et al., 2019) **(Figure 48 and Supplementary Table 7**). It is downregulated during *U. maydis* infection (Kretschmer et al., 2017). Ding et al. (2019), found that this gene is downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi.

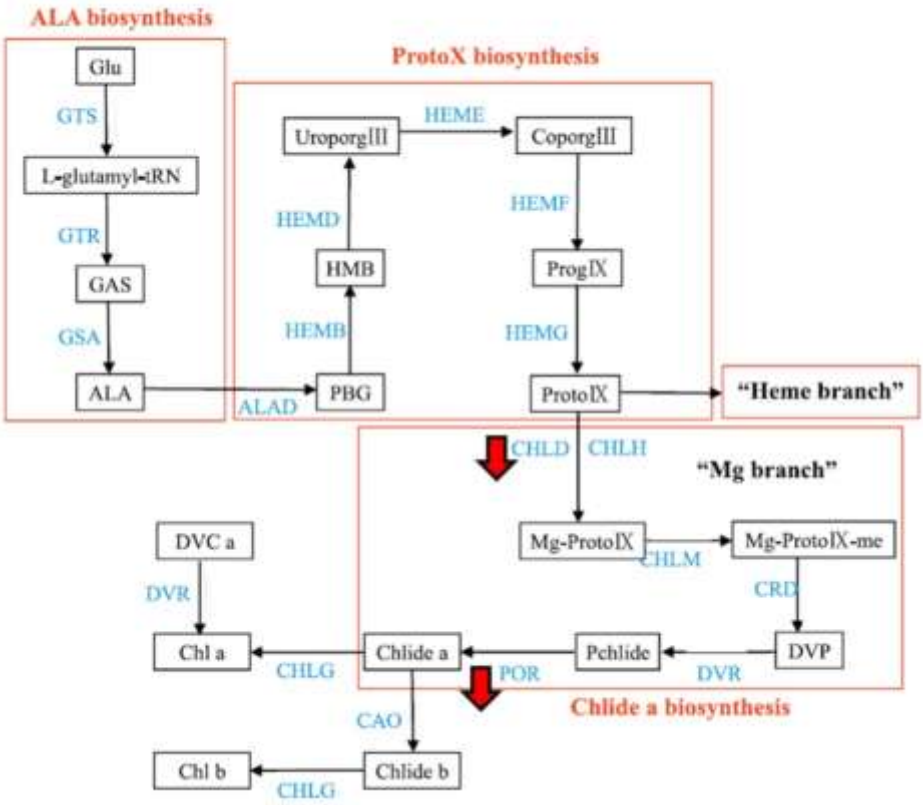


Figure 48. CgEP3 effector may modulate chlorophyll biosynthesis pathway. Abbreviated scheme of chlorophyll biosynthesis pathway in maize plants. Capital letters in blue are the names of several enzymes that contribute to biosynthesis. CgEP3 could suppress synthesis of two important enzymes: CHLD and PORA. Red arrows with black borders represent genes that are suppressed by CgEP3. This figure has been modified from the original that is found in the article by Zhang et al. (2019).

Related to photosystems and electron transfer chain

The chloroplasts possess an internal network of membranous compartments called thylakoid. These contain the machinery required for the production of chemical energy that is indispensable to the Earth's life. This machinery is formed by photosystem II (PSII), cytochrome b6f, photosystem I (PSI), and ATP synthase, which play a role in the light reaction of photosynthesis. There is a linear electron transfer chain composed of the interconnection of both photosystems by the cytochrome b_6f complex, and also by the two mobile electron carriers, plastoquinone and plastocyanin, in the lipid bilayer and thylakoid lumen, respectively. The energy of the sun (light) is caught by light-harvesting complexes (LHCs), which are linked to the photosystems. Then, thylakoidal protein complexes carried out the transfer of electrons and protons, which produce chemical energy (conserved as molecules of NADPH and ATP), which can be utilized later in future reactions, such as the fixation of carbon (during the dark reaction) (Rantala et al., 2020).

The maize gene *ZmLHCBa* (*GRMZM2G351977*) (Dubreuil et al., 2018) (**Figure 49 and Supplementary Table 7**), based on *A. thaliana* studies, encodes a light-harvesting chlorophyll a/b protein of photosystem II (Allen et al., 2011; Zhenfeng Liu et al., 2004; Standfuss et al., 2005). Ding et al. (2019) found that this gene is downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. Homologs of this gene in rice (*LOC_Os01g41710.1*), in the study by Vergne et al., (2007), were downregulated during the early stages of *M. oryzae* infection at 24 hpi. This suggests that down-regulation of chlorophyll a/b binding genes is associated with the onset of plant defense mechanisms. In a study by Zhi-ming et al. (2021), this gene was downregulated against *R. solani* infection.

The maize gene *GRMZM2G036880* is annotated as light-harvesting complex I chlorophyll a/b binding protein 1 (LHCA1) (Kanehisa et al., 2016) **(Figure 49 and Supplementary Table 7)**. Based on *A. thaliana* studies, homologous genes participate in photosystem I (Rantala et al., 2020). Yang et al. (2018), found that this gene is downregulated against the hemibiotrophic fungus *Exserohilum turcicum* (causing northern corn leaf blight) in maize resistant W22Htn1 y B73Htn1 lines at 9 hpi. In the thesis dissertation of Bruns (2015), this gene was reported to be upregulated against *Fusarium graminearum* maize infection at 84 hpi. Buffon et al. (2016) showed that for homologs genes in rice (*LOC_Os06g21590.1*), this protein was identified in rice leaves under early infested conditions with the mite (*Schizotetranychus oryzae*). Cartieaux et al. (2008) reported that homologs of this gene in *A. thaliana* (*AT3G54890.1*) is suppressed during infection with *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000). Jia et al. (2020), reported that this gene was upregulated in plants that were infected with *Pst* DC3000, which were previously treated with chitosan oligosaccharides (COS). COS acted efficiently in the regulation of defense responses against several plant pathogens; however, because the signaling network activated by COS is very complex, further research is needed to understand the exact mechanism of regulation. This study identified proteins that are related to the acquired resistance by COS against the pathogen *Pst* DC3000 in *A. thaliana* (X. Jia et al., 2020). LHCA1 was highly increased after pretreatment of plants with COS. Thus, COS may have a positive effect on photosynthesis and can improve the efficiency of photosynthesis during plant infections. Molho et al. (2021), determined that the replication protein p33 of tomato bushy stunt virus can interact with LHCA1 in a membranous environment. Protein-protein screening can enrich the knowledge of how tombusviruses can benefit from the use of host proteins and alteration of host pathways. The thesis dissertation of Le (2012) indicated that this protein interacted with the intracellular kinase domain of the receptor AtCERK1. It was identified using two yeast hybrid assays. AtCERK1 is a plasma membrane receptor that recognizes fungal chitin as PAMP, a component of fungal cell walls. AtCERK1 can trigger a PTI plant defense response. Ali et al. (2017) showed that this gene is upregulated against the nematode *Heterodera schachtii* during root infection.

The maize gene *GRMZM2G450705* is annotated as NAD(P)H-quinone oxidoreductase subunit 2 (NdhB) (Kanehisa et al., 2016) **(Figure 49 and Supplementary Table 7)**. NDHB participates in NADH dehydrogenase-like complex type 1 (NDH-1) (Laughlin et al., 2020). NDH possesses 11 of the 14-core complex subunits, as well as several oxygenic-photosynthesis-specific subunits that are conserved from cyanobacteria to plants (Laughlin et al., 2019; Peltier et al., 2016; Shikanai, 2016). It plays a role in NAD/NADH phosphorylation and dephosphorylation in aerobic respiration I and III (in the alternative oxidase pathway) (Portwood et al., 2019). Mata-Pérez et al. (2015) reported that homologs of the gene in *A. thaliana* (*ATCG00890.1*) participates in the NDH complex and is downregulated against herbivore attacks. This fact can help plants drive their resources towards defense responses among others (Halitschke et al., 2001; Hui et al., 2003; Mata-Pérez et al., 2015).

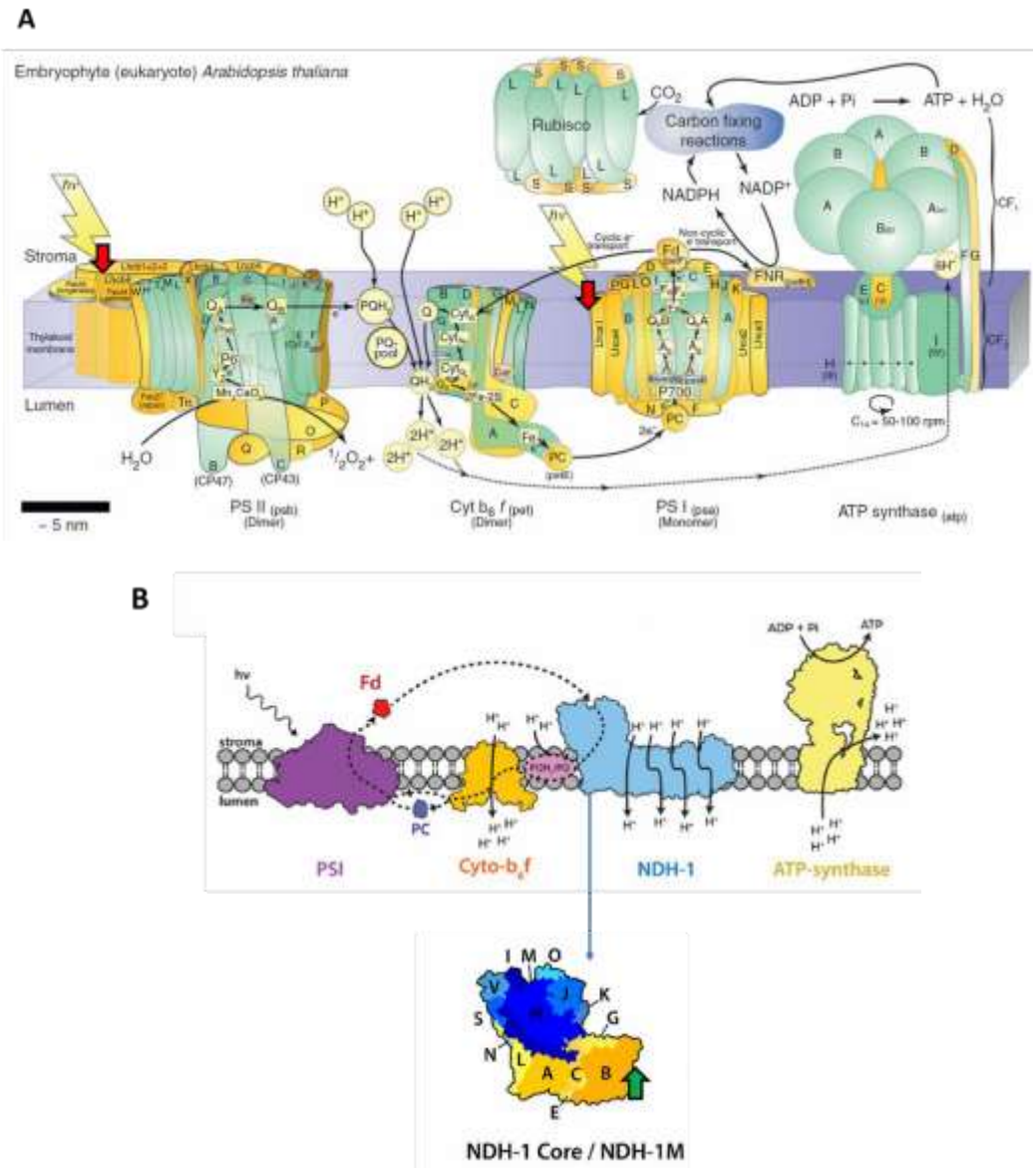


Figure 49. CgEP3 could modulate plant photosystems and electron transfer chains. A) Scheme of the main complexes and proteins that are found in the photosynthetic apparatus of chloroplasts of the plant model *A. thaliana*: The photosystems I and II (PS I and PS II respectively), cytochrome b6f (Cyt b6f), ATP synthase, and Rubisco. CgEP3 could suppress two important components of PSII, LHCBa, and PSI, LHCA1. Red arrows with black borders represent genes that are suppressed by CgEP3. This figure has been modified from the original that is found in the article by Allen et al. (2011). **B) (**Above) Scheme of complexes that form the electron transport chain. It shows the transfer of electrons, translocation of protons, and the ATP synthase which synthesizes ATP. Dashed lines are the paths followed by electrons, from the PSI through different carriers until returned again to PSI. (Below) scheme in detail of NDH-1 core complex medium (NDH-1M) based on cyanobacterial structure. CgEP3 could induce one component of NDH-1, NdhB. Green arrow with black border represents the gene that is induced by CgEP3. This figure has been modified from the original that is found in the article by Laughlin et al. (2020).

The maize gene *GRMZM2G070847*, in the study of Tang et al. (2018), was annotated as a DNA translocase and it had an enrichment in GO terms, such as "P: vesicle-mediated transport; C: plastid; C: thylakoid" **(Figure 50 and Supplementary Table 7)**. They observed that this gene was downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi (Y. Ding et al., 2019). The thesis dissertation of Han (2017) reported that homologs of this gene in *A. thaliana* (*AT1G21500.1*) were annotated as phosphatidylinositol 3-kinase class IA (PI3KIA). He predicted that it is a plastidial kinase that is involved in the regulation of starch biosynthesis. In

the study by Hao et al. (2020), the protein encoded by this gene was shown to be localized in the thylakoid lumen. Through two yeast hybrid assays, it was discovered that it can interact with AtCYP38 (Cyclophilin 38), which is associated with PSII. AtCYP38, which is also located in the thylakoid lumen, can help in early PSII biogenesis, proper assembly, and maintenance (Sirpiö et al., 2008). In the thesis dissertation of Spielau (2010), the phosphatidylinositol protein kinase (AT1G21500.1) was shown to accumulate in MPK6-strepII plants after flagellin22 (flg22) treatment. MPK6-strepll plants constitutively express the protein kinase MPK6, which is activated after flg22 treatment and triggers a defense response. Through affinity chromatography, she identified proteins that coelute with MPK6 and then identified the kinases that are involved in pathogen defense.

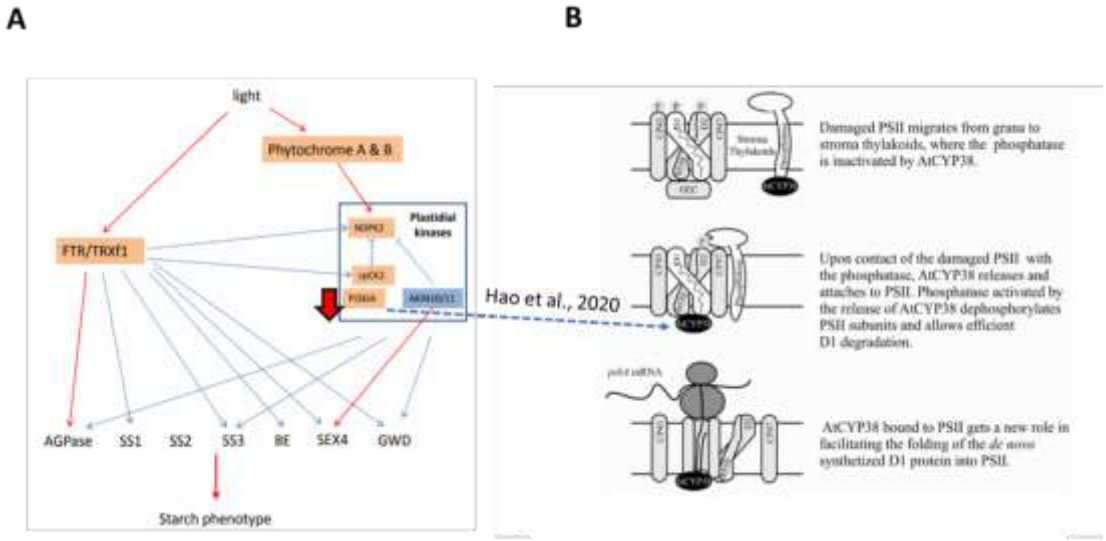


Figure 50. CgEP3 could modulate starch metabolism and interfere with PSII damage repair. A) Schematic regulation of starch biosynthesis. CgEP3 could suppress plastidial protein kinase PI3KIA. Red arrow with black border represents the gene that is suppressed by CgEP3. This figure has been modified from the original that is found in the thesis dissertation of Han, (2017). **B)** Hypothetical model of the dual function of AtCYP38 in PSII repair. Hao et al. (2020), has discovered that this protein kinase can interact with AtCYP38 which is involved in PSII damage repair. CgEP3 could affect this process. This figure has been modified from the original that is found in the thesis dissertation of Sirpiö (2009).

GENES RELATED TO METABOLISM

Plants interact constantly with the environment and microorganisms, and consequently, have developed complex mechanisms to identify them and trigger appropriate defense responses against the pathogens. Essential processes for plant growth and development, such as photosynthesis and primary metabolism, are downregulated during interactions with pathogens. The energy that the plants conserve from these processes can be utilized for the plant immune responses. However, diverse studies have also reported that primary metabolism is also upregulated during pathogen infection. Thus, primary metabolism can control different signal transduction cascades that allow plant defense responses (Rojas et al., 2014a). We found several genes that belong to metabolic processes that could be affected by CgEP3. These genes are related to sulfur metabolism (*GRMZM2G088348* and *GRMZM2G342907* - both induced), selenium metabolism (*GRMZM2G058930* - suppressed), carbon metabolism (*GRMZM2G446253* and *GRMZM2G439389* - both suppressed), lipid metabolism (*GRMZM2G062531* - suppressed), and iron metabolism (*GRMZM2G132875* and *GRMZM2G172239* - both induced) **(Supplementary Table 7, Figures 51, 52 53 and 54)**.

Related to sulfur metabolism

Macronutrient sulfur (S) is fundamental for plant growth and development (Chorianopoulou et al., 2020). S plays a role in several processes, such as the transport and regulation of electrons, production of oxygen during photosynthesis, and resistance to biotic and abiotic stresses (Capaldi et al., 2015). S is incorporated into the plant bodies primarily from the soil as sulfates through the S-transporters. They biosynthesize organic molecules that contain sulfur, such as amino acids of proteins (cysteine and methionine), co-enzymes, different prosthetic groups, and molecules derived from secondary metabolism (Bohrer & Takahashi, 2016; Chorianopoulou et al., 2020; H. Takahashi et al., 2011). Certain compounds help plant subsist and improve the defense response; they are known as sulfur-containing defense compounds (SDCs), and they include elemental sulfur (S^0), glutathione (GSH), glutathione-S-transferase, phytochelatins, proteins that are enriched in S, and several secondary metabolites (Capaldi et al., 2015; X. Fu et al., 2016; Rausch & Wachter, 2005). An example of the importance of S during fungal infection is described in the work of Fu et al. (2016). They observed that genes that play a role in S absorption and SDCs were upregulated during infection with *Verticillium dahliae* in tomato plants. However, the role of S in resistance against this fungus remains unclear (X. Fu et al., 2016). S is also implicated in two different phenomena: S-induced defense, when plants respond to pathogens, and S-induced resistance, when plants respond to abiotic stresses. In both cases, the principal consequence is the increase in S compounds, which can help alleviate the stress. For example, the generation of S^0 and sulfide (S^{2-}) during the infection of *Verticillium* and *Fusarium* in tomato increases plant resistance (Williams et al., 2002a; Z. L. Zheng & Leustek, 2017). On the other hand, S compounds can play a vital role in detoxification during high concentration of ROS, and against heavy metal stresses. For example, GSH is necessary to alleviate stress (Capaldi et al., 2015; Noctor et al., 2012; Z. L. Zheng & Leustek, 2017). Finally, several studies have demonstrated the relationship between plant hormones and S absorption (Capaldi et al., 2015; Kopriva, 2006; Maruyama-Nakashita et al., 2004, 2005). Plant hormone signaling pathways are related to plant defense and efficient utilization of nutrients, among others (Capaldi et al., 2015; Fatma et al., 2013). CgEP3 could induce two genes related to sulfur metabolism: *GRMZM2G342907* and *GRMZM2G088348* **(Supplementary Table 7 and Figure 51)**.

In maize, based on studies of protein homology in *A. thaliana, the GRMZM2G342907* gene encodes a putative sulfate transport protein, ZmSULTR1.2b **(Supplementary Table 7 and Figure 51)**. According to phylogenetic analyses it belongs to group 1 of *A. thaliana* sulfur transport system (Chorianopoulou et al., 2020). The transporters of this group have a strong affinity for sulfate and are found in the roots. It participates in the soil sulfate absorption (Chorianopoulou et al., 2020; H. Takahashi et al., 2000; Vidmar et al., 2000; Yoshimoto et al., 2002, 2007). If we study the new data on homologous genes in the Zm-B73-Reference-Gramene-4.0 maize genome (AGPv4), it corresponds to *Zm00001d048189* (Chorianopoulou et al., 2020; Y. Jiao et al., 2017), and is induced against biotic and abiotic stresses. In *C. graminicola* infection, *Zm00001d048189* increased its expression in leaves at 48 hpi. It is also modulated by *C. zeina* infection in the lower leaves of maize plants (Hoopes et al., 2019).

In maize, *GRMZM2G088348* is predicted to be ATP sulfurylase (ATPS). It participates in the conversion of sulfate in adenosine 5'-phosphosulfate through the sulfate reduction pathway (EC: 2.7.7.4) (Portwood et al., 2019) **(Supplementary Table 7, Figure 51)**. Homologs of this gene in rice (*LOC_Os03g53230.1*) encodes an ATPS that is involved in sulfur metabolism (Zhang et al., 2016). ATPS can act together with other sulfur metabolizing genes as a sensor for sulfur limitation and constitutes an integral part of the regulatory network of sulfate assimilation in

plants (Smita Kumar et al., 2011; G. Liang et al., 2010; Matthewman et al., 2012; D. Sharma et al., 2014). Proteomic analysis by Zhang (2014) showed that the protein LOC_Os03g53230.1 was significantly upregulated after fall armyworm (*Spodoptera frugiperda*) herbivory. Homologs of this gene in *A. thaliana, AT5G43780.1*, encodes an ATPS4, which is part of the chloroplast proteome (Mcwilliams, 2007). The ATPS enzyme is crucial for the reduction of sulfate in plants (Akbudak & Filiz, 2019a). This gene is upregulated against *Botrytis cinerea* infection at 18 hpi (Whitley, 2019). Likewise, ATPS function can be triggered by oxidative stress in the roots of rapeseed plants (*Brassica napus*) (Akbudak & Filiz, 2019a; Lappartient & Touraine, 1997).

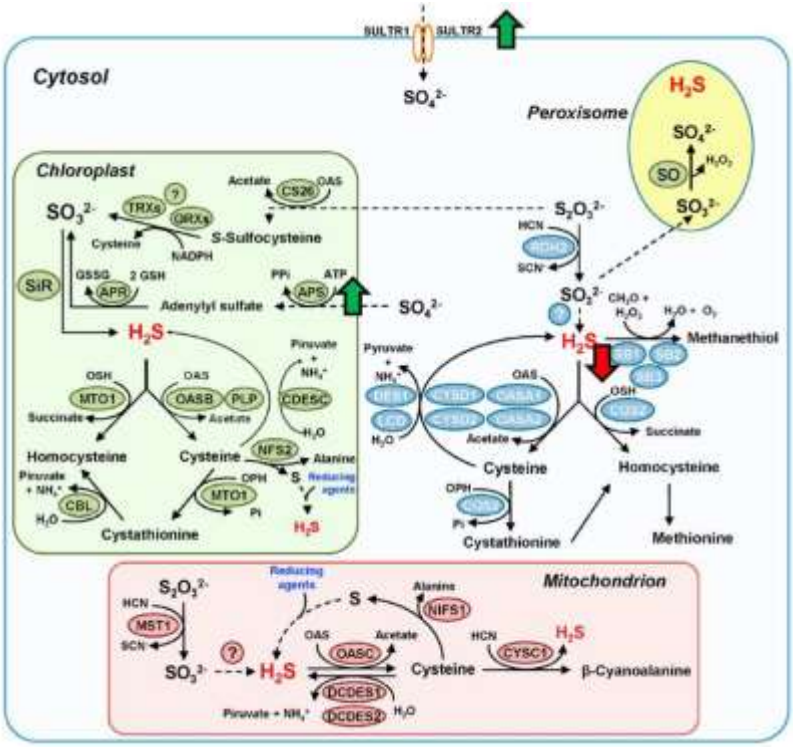


Figure 51. CgEP3 effector could modulate sulfur and selenium metabolism. Scheme of H_2S metabolism through cysteine (Cys) metabolic pathways and reactions occurring in distinct cellular compartments (e.g., cytosol, chloroplast, peroxisome, and mitochondrion) of the model plant *A. thaliana*. CgEP3 could induce the expression of sulfur transporter and ATP sulfurylase. Sulfur metabolism is related with selenium metabolism through selenium binding proteins which act in detecting the redox disequilibrium in the cell (Valassakis et al., 2018). CgEP3 could suppress SBP1 and affect selenium metabolism. This figure has been modified from the original that is found in the article by González-Gordo et al. (2020). Red and green arrows with black borders represent genes that are suppressed or induced respectively by CgEP3.

Related to selenium metabolism

Selenium is an element that is close to sulfur because of its chemical features. Primary metabolism is shared between these two elements. Selenium is found in diverse compounds and can be linked to carbon through covalent bonds. These compounds are known as organoselenium compounds. Selenoproteins are important for the physiology of a variety of organisms, such as prokaryotes, archaebacteria, and eukaryotes. However, these are not found in plants and fungi (Castellano et al., 2004; Cognat et al., 2012; Santesmasses et al., 2017; Schomburg & Arnér, 2017; P. J. White, 2018). The majority of selenoproteins are enzymes that are implicated in redox reactions that utilize selenocysteine and cysteine residues in their active sites (Reich & Hondal, 2016; Schomburg & Arnér, 2017; P. J. White, 2018). However, although selenium is not present in flowering plants, several studies have shown that it is important for

plant subsistence and development under certain conditions (El Mehdawi & Pilon-Smits, 2012; Feng et al., 2013; Pilon-Smits et al., 2009; Schiavon & Pilon-Smits, 2017; P. J. White, 2016, 2018). CgEP3 modulated selenium metabolism by suppressing *GRMZM2G058930* **(Supplementary Table 7 and Figure 51)**.

The maize *GRMZM2G058930* gene is annotated as a selenium binding protein, SELENBP1 (methanethiol oxidase, EC:1.8.3.4) (Kanehisa et al., 2016) **(Supplementary Table 7 and Figure 51)**. The homolog of this gene in rice (*LOC_Os01g68770.1*) encodes OsSBP. Rice plants that constitutively express the *OsSBP* gene improved the resistance against the fungus *Magnaporthe oryzae* and the bacteria *X. oryzae* pv. *oryzae,* causing rice blast and rice bacterial blight diseases, respectively. The same plants infected with *M. oryzae* also showed increased expression of defense genes and phytoalexins. Then, the OsSBP protein could participate in encouraging the defense response against biotic stresses in rice (Sawada et al., 2004). Homologs of this gene in *A. thaliana* (*AT4G14030.1*) have been reviewed in the work of González-Gordo et al. (2020). SBP1 plays a role in detecting redox disequilibrium in cells (Valassakis et al., 2018), selenium toxicity (Agalou et al., 2005), when sulfur sources are required by plant cells (Hugouvieux et al., 2009), etc. Therefore, the *SBP1* gene may be implicated in general stress responses in *A. thaliana* (Dervisi et al., 2020; Nikiforova et al., 2003).

Related to carbon metabolism

Sugars play an important role in regulating the life cycle of plants, controlling their growth and development, and interacting with phytohormones (Morkunas & Ratajczak, 2014; Stokes et al., 2013; Wind et al., 2010). Sugars are important for plant immunity as they offer energy to plants, participate as signaling molecules, and are also involved in the regulation of defenses. During the early stages of infection, sugars can increase oxidative burst, lignification of plant cell walls, and stimulate the biosynthesis of pathogenesis-related proteins or flavonoids (Morkunas & Ratajczak, 2014). CgEP3 suppressed two genes of the glycolysis pathway: *GRMZM2G446253* and *GRMZM2G439389* **(Supplementary Table 7 and Figure 52)**.

The maize gene *GRMZM2G446253* was annotated as enolase (Kanehisa et al., 2016) **(Supplementary Table 7 and Figure 52)**. Ding et al. (2019) found that this gene is upregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. In rice, homologs of this gene (*LOC_Os06g04510.1*) is downregulated when plants are infected with the virus rice black-streaked dwarf virus (Ahmed et al., 2017). This gene is also related to rice sheath blight caused by *R. solani.* In this study, it was defined as a candidate resistance gene against this pathogen (Fan Zhang et al., 2019). Another study demonstrated the interaction of this gene with virulence: enolase metabolic protein was upregulated against the pest fall armyworm (*S. frugiperda*). It is related to the biosynthesis of pyruvate and the downstream biosynthesis of secondary metabolites (Yixiang Zhang et al., 2013). The thesis dissertation by Liu (2015) reported how homologs of this gene in *A. thaliana* (*AT2G36530.1*) responds to flg22 elicitation. These cysteine-containing proteins were found to be redox-sensitive in response to flg22 treatment. Kaffamik et al. (2009), have shown that this protein of primary metabolism is induced against *P. syringae* pv. *tomato* DC3000. Another study by Mulema et al. (2011), reported that enolase protein was present in fractions of *A. thaliana* leaf tissues and there was a decrease in absorbance when plants were infected with *B. cinerea* after 12 hpi.

The maize gene *GRMZM2G439389* is predicted to putatively express cytosolic triosephosphate isomerase (TPI) (Goodstein et al., 2012) **(Supplementary Table 7 and Figure 52)**. Homologs of this gene in rice (*LOC_Os01g62420.1*) are found in the cytoplasm. The TPI enzyme participates

in the conversion of d-glyceraldehyde 3-posphate to glycerone phosphate. This enzyme is also important in other metabolic pathways, such as gluconeogenesis, fatty acid synthesis, the pentose phosphate pathway, and carbon dioxide assimilation through photosynthesis. It is expressed in response to several stresses: either induced by wounding (at 6–24 h after treatment (hat)), low temperature and H_2O_2 stresses (6 hat), or suppressed under heat, drought, and NaCl (6–24 hat) stresses (S. Sharma et al., 2012). The homologous gene of maize in *A. thaliana* (*AT3G55440.1*) is involved in primary metabolism and is induced by *P. syringae* pv. *tomato* DC3000 (Kaffamik et al., 2009). Wang et al. (2012), determined that this protein could be important for redox sensitivity during early response after H_2O_2 treatment. After studying oxidative stress in the yeast model system, it was shown that the inhibition of glycolysis could be due to the metabolic flux directed towards the pentose phosphate pathway. The generated NADPH is used by the antioxidant system of the cells (Ralser et al., 2007; Shenton & Grant, 2003; H. Wang et al., 2012).

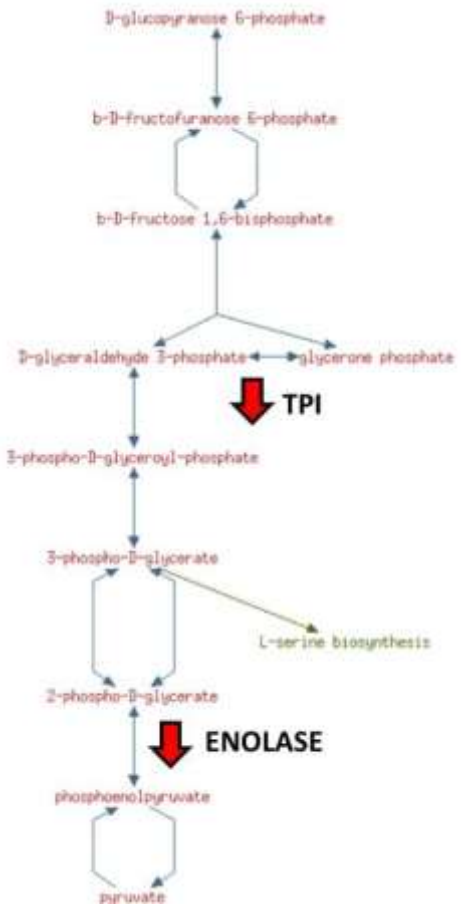


Figure 52. CgEP3 could modulate carbon metabolism. Enzymatic reactions involved in maize glycolysis. CgEP3 could suppress two important enzymes of glycolytic pathway: enolase and triosephosphate isomerase (TPI) affecting carbon metabolism. Red arrows with black borders represent genes that are suppressed by CgEP3. This figure has been modified from the original that is found in the database of MaizeGDB (Portwood et al., 2019).

Related to lipid metabolism

Lipids are a diverse group of molecules that are essential for the development of cells as well as the responses of plants to biotic and abiotic stresses (Rojas et al., 2014a; Welti et al., 2007). They can fulfill several functions, such as structural maintenance of cell walls and cell membranes, supplying energy for metabolism, signal transduction, membrane trafficking, and cytoskeletal rearrangements (Rojas et al., 2014a; Xuemin Wang, 2004; Welti et al., 2007). CgEP3 suppressed one gene involved in phytosterol biosynthesis, *GRMZM2G062531* **(Supplementary Table 7 and Figure 53)**.

The maize gene *GRMZM2G062531* is annotated as plant 4,4-dimethysterol C-4 alpha-methyl-monooxygenase (SMO1) (Kanehisa et al., 2016) **(Supplementary Table 7 and Figure 53)** and belongs to the fatty acid hydroxylase (FAH) superfamily (Dutta et al., 2019). The FAH superfamily includes a broad spectrum of proteins, including carotene, FAHs, and sterol desaturases in plants. Within this group, sterol desaturase and C-4 sterol methyl oxidase were found (Dutta et al., 2019). Sterols are structurally lipophilic molecules found in the majority of eukaryotic cell membranes (Gulati et al., 2010). Sterols and phospholipids are responsible for preserving membrane fluidity (Lagace & Ridgway, 2013). Lambarey et al. (2020) showed that this gene is upregulated during *Fusarium verticillioides* infection in a susceptible African maize line. Another example is the study of Ding et al. (2019), where this gene is downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi.

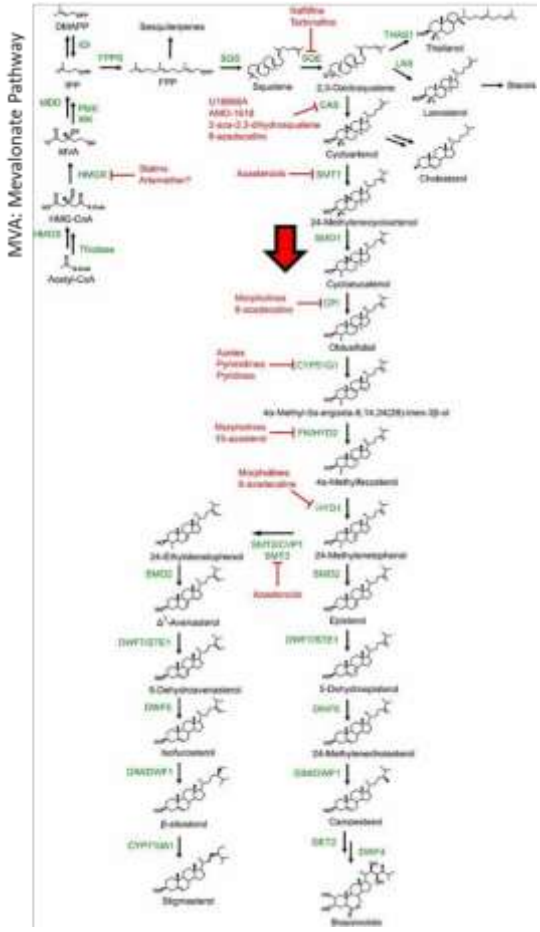


Figure 53. CgEP3 could modulate lipid metabolism through phytosterol biosynthesis pathway. Scheme of the principal sterol biosynthesis pathway in *A. thaliana* and putative target sites of some inhibitors. CgEP3 could suppress SMO1 enzyme, which is important for phytosterol biosynthesis. Red arrow with black border represents the genes that are expressed by CgEP3. This figure has been modified from the original that is found in the article by de Vriese et al. (2019).

Related to iron metabolism

Iron is an important element for a large number of organisms. Pathogens and hosts often compete for this nutrient. The role of iron during host-pathogen interaction is intricate. Iron can be used by plants to increase oxidative stress during the defense response, the liberation of phenolic compounds, etc. Certain components, such as iron transporters or iron storing proteins, help in the specific localization of iron and the defense response during infection. However, the role of iron depends on the host and the strategy of infection followed by the

pathogen (Aznar et al., 2015) . CgEP3 could modulate two genes related to iron metabolism, including *GRMZM2G132875* and *GRMZM2G172239* **(Supplementary Table 7 and Figure 54)**.

In maize, the *GRMZM2G132875* gene encodes a 3”-deamino-3’’-oxonicotianamine reductase (DMAS1) (Kanehisa et al., 2016) which plays a role in the biosynthesis of the family of phytosiderophores (PS) of mugineic acid (Aznar et al., 2015; Portwood et al., 2019) **(Supplementary Table 7, and Figure 54)**. Members of *Poaceae* release PS chelators of Fe (III) from the roots to gain iron from the soil. This is known as strategy II (Aznar et al., 2015). Meyer et al. (2017) found that this gene is downregulated during *C. zeina* infection in maize. Homologs of this gene in rice (*LOC_Os04g37490.1*) is predicted to be correlated with the partial resistance of rice against *M. oryzae* infection (Grand et al., 2012).

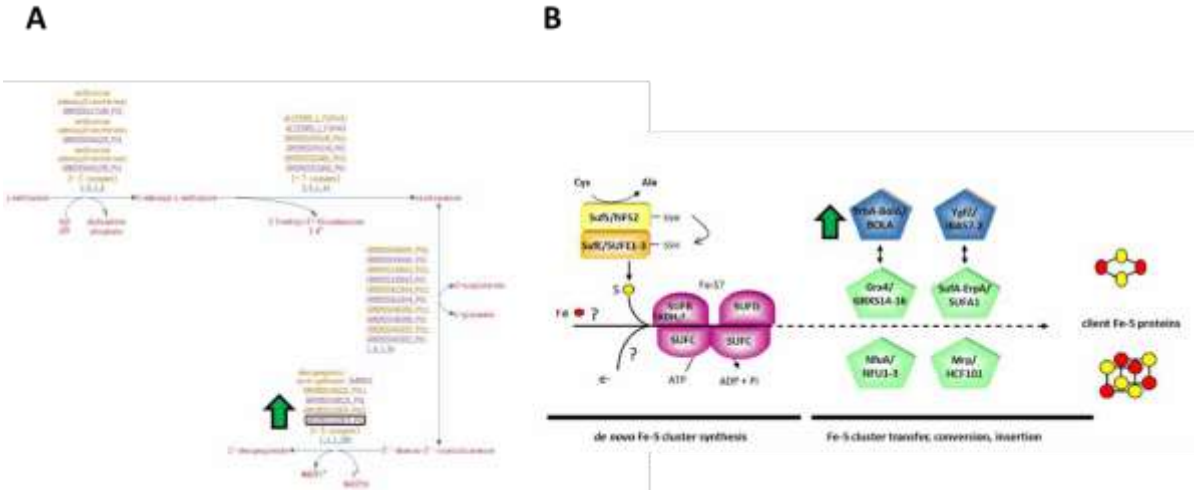


Figure 54. CgEP3 could modulate iron metabolism. A) Pathway for 2'-deoxymugineic acid phytosiderophore biosynthesis in maize. CgEP3 could induce the expression of the enzyme 3''-deamino-3''-oxonicotianamine reductase which catalyzes the last step of the pathway from 3”-deamino-3”-oxonicotianamine to 2’deoxymugineate. This figure has been modified from the original that is found in the database of MaizeGDB, Portwood et al. (2019). **B)** Scheme of operation of the sulfur mobilization protein machinery system (SUF). The names of the *Escherichia coli/ A. thaliana* proteins are shown (except when they were similar). In yellow/orange, the sulfur (S) delivery system; in violet, the iron-sulfur (Fe-S) cluster scaffold complex; in green, the Fe-S cluster transfer proteins; and in blue, associated targeting factors (like BOLA). NFU and HCF101 participates in the maturation of (4Fe-4S)-containing client proteins, and GRX and SUFA contributes to the maturation of (2Fe-2S)-containing client proteins. CgEP3 could induce the BOLA targeting factor in this pathway. This figure has been modified from the original that is found in the article by Przybyla-Toscano et al. (2021). In both cases, green arrows with black borders represent genes that are induced by CgEP3.

Maize *GRMZM2G172239* is annotated as BolA-like protein 1 (BOLA1) (Kanehisa et al., 2016) **(Supplementary Table 7 and Figure 54)**. Homologs of this gene in *A. thaliana* (*AT1G55805.1*) encode a plastid BOLA1 protein (Couturier et al., 2014) which is a targeting factor involved in iron-sulfur (Fe-S) cluster trafficking. Proteins that contain iron in their structure, as well as iron-sulfur proteins, play an important role in many metabolic and electron transfer reactions. They are present in the majority of cell compartments, such as plastids, where they participate in the electron transport chains of photosynthesis. In addition, iron-sulfur proteins contribute to the fixation of various elements, such as carbon, nitrogen, and sulfur; the synthesis of thiamine and lipoic acid; and the metabolism of tetrapyrroles and isoprenoids (Przybyla-Toscano et al., 2021). BolA-like proteins may control redox reactions (Couturier et al., 2014; Mostafa et al., 2016). In turn, the activity of BolA-like proteins is controlled by the interaction of monothiol glutaredoxins found in the same cell compartments (Couturier et al., 2014; L. Qin et al., 2015). In the study by Whitley (2019), this gene is reportedly downregulated during *B. cinerea* infection at 18 hpi.

GENES RELATED TO SECONDARY METABOLISM

Plants generate a great variety of low molecular weight compounds, including secondary metabolites (Erb & Kliebenstein, 2020; Taiz et al., 2015). Secondary metabolites often belong to a specific plant lineage and help plants interact with biotic and abiotic environments. The most abundant metabolites belong to the group of phenols, terpenes, and compounds that contain nitrogen (Erb & Kliebenstein, 2020; T. Hartmann, 2007). Secondary metabolites fulfill important functions, such as aiding in growth and development, and the defense response of plants (Erb & Kliebenstein, 2020). We found several genes related to the secondary metabolic process that could be modulated by CgEP3. The genes *GRMZM2G424857*, *GRMZM2G085924*, and *GRMZM2G066291* are related to the phenylpropanoid pathway and are suppressed by CgEP3, whereas the gene *GRMZM2G127087* is related to the MEP pathway and biosynthesis of sesquiterpenoid phytoalexins and is induced by CgEP3 **(Supplementary Table 7, Figures 55, 56, and 57)**.

Related to phenylpropanoid pathway

Phenylpropanoid metabolism is important in plants. Plants can produce more than 8000 different metabolites that help in their growth and development, as well as their interaction with the environment. The phenylpropanoid pathway contains numerous branches with products such as flavonoids, lignin, phenylpropanoid esters, lignans, hydroxycinnamic acid amides, and sporopollenin (N. Q. Dong & Lin, 2020). When a pathogen reaches the plant cell wall, the phenylpropanoid pathway is triggered as a plant defense response. In turn, when the pathogen is capable of breaking the plant cell wall, a great variety of compounds are produced that are related to a few intermediaries in the shikimate pathway. The phenylpropanoid pathway is finely regulated at the transcriptional, post-transcriptional, and post-translational levels involving multiple gene families. These genes generate products that can act as antimicrobial or signaling molecules (V. Yadav et al., 2020).

The maize gene *GRMZM2G066291* is annotated as Phytochrome Kinase Substrate 1 (PKS1), which promotes flowering (E. Johnson et al., 1994; Reed et al., 1994; K. Song et al., 2017) **(Supplementary Table 7 and Figure 55)**. Ding et al. (2019) found that this gene is downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. The thesis dissertation of Pan (2012) relates homologs of this gene in *A. thaliana* (*AT1G18810.1*) to a subnetwork of the phenylpropanoid pathway, particularly with naringenin-chalcone synthase (CHS), which is important in flavonoid biosynthesis.

In maize, *GRMZM2G424857* encodes a protein from the acetamidase/formamidase family (ATD) (Wen et al., 2014) **(Supplementary Table 7 and Figure 55)**. Plants produce a wide variety of metabolites that can participate in plant stress responses. ATD participates in flavonoid biosynthesis in maize kernels. It could be involved in the formation of hesperetin from naringenin (Wen et al., 2014).

In maize, the *GRMZM2G085924* gene is annotated as an o-methyltransferase protein (OMT) (Liu et al., 2019) **(Supplementary Table 7, Figure 5)**. As per MaizeGDB (Portwood et al., 2019), it could be implicated in flavonoid biosynthesis, particularly, salvigenin biosynthesis. Plant OMTs are dependent on S-adenosyl-L-methionine (SAM), which can generate highly diverse secondary metabolites, such as flavonoids, alkaloids, and phytoalexins. They can participate during the

growth and development of plants, as well as resistance against pathogens (Miao et al., 2016). In a study by Gao et al. (2014), this gene is reported to be downregulated during *R. solani* infection, which causes leaf and sheath blight diseases in maize. It has been proposed that o-methyltransferase plays a role in plant immunity against pathogens (Gao et al., 2014; He et al., 2010). Another example is the study by Ding et al. (2019), where this gene was found to be downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi.

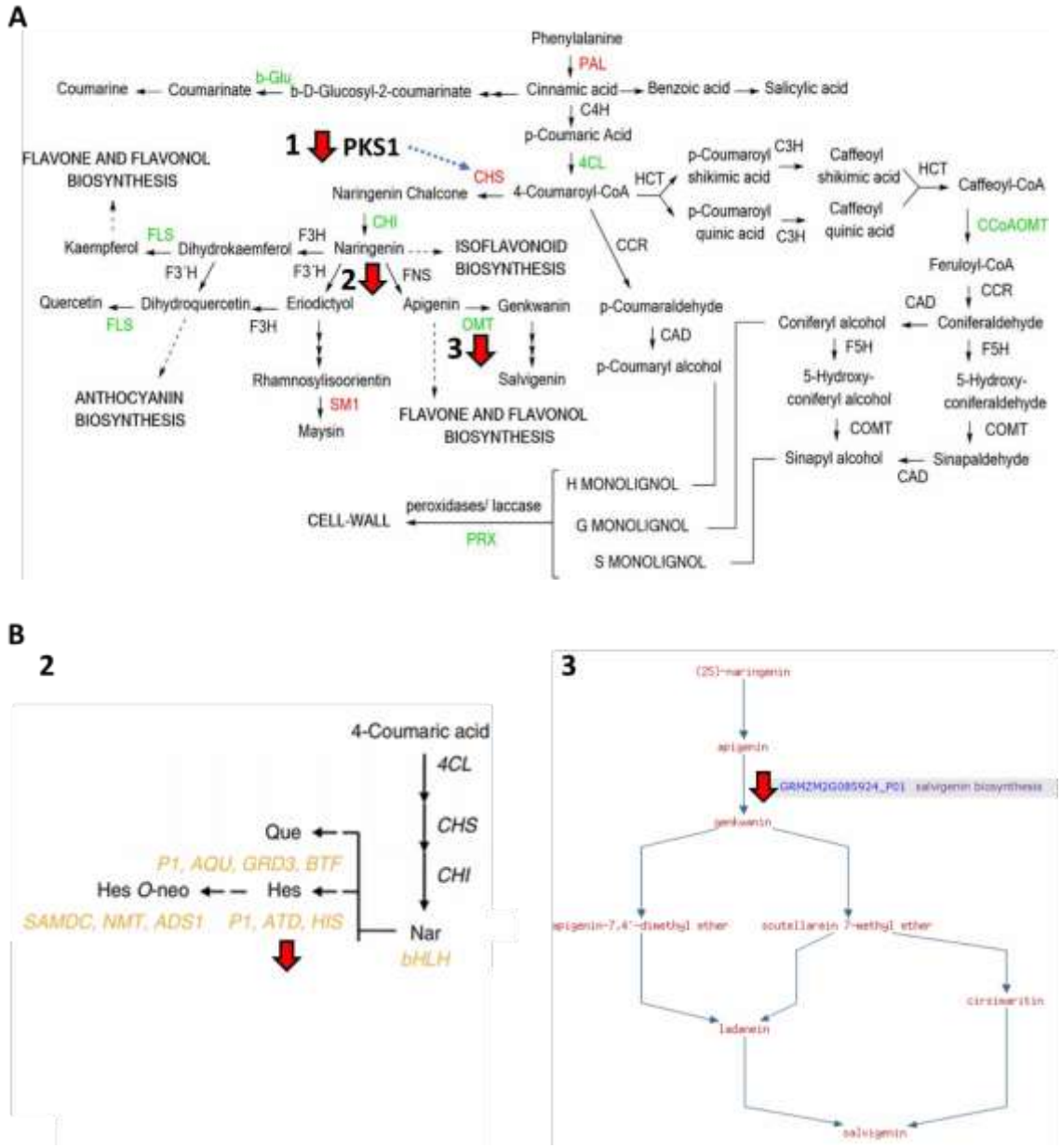


Figure 55. CgEP3 effector could modulate secondary metabolism through phenylpropanoid pathway. A) The phenylpropanoid pathway in maize. CgEP3 could suppress the expression of **1)** PKS1 which could be related with naringenin-chalcone synthase (CHS) (Pan, 2012); **2)** protein from acetamidase/formamidase family (ATD) which is related to the formation of hesperetin from naringenin; and **3)** o-methyltransferase protein (OMT) which is related to the biosynthesis of salvigenin from naringenin. This figure has been modified from the original that is found in the article by Agostini et al. (2021). **B)** Magnified view of the **s**teps of the phenylpropanoid pathway in detail which could be affected by CgEP3. Further information is provided in points A2 and A3. These figures have been modified from the original that are found in the article by Wen et al. (2014) (B2) and in the database MaizeGDB, Portwood et al. (2019) (B3). In all cases, red arrows with black borders represent genes that are suppressed by CgEP3.

Related to MEP pathway and sesquiterpenoid biosynthesis

In maize, *GRMZM2G127087* encodes the enzyme terpene synthase 6 (TPS6) **(Supplementary Table 7, Figures 56 and 57)**, which is related to secondary metabolism. TPS6 is elicited in response to several fungal pathogens, as described in a review by Block et al. (2019). TPS6, together with another redundant enzyme (TPS11), and geranyl diphosphate, helps to a certain extent in the production of either acyclic monoterpenes (β-myrcene and linalool), or cyclic monoterpenes (limonene, α-thujene, sabinene, and α-terpinolene). However, both enzymes generate monocyclic sesquiterpenes (β-bisabolene and β-macrocarpene) in the presence of farnesyl diphosphate. β-macrocarpene participates in the biosynthesis of zealexins (Huffaker et al., 2011; Köllner et al., 2008). Zealexin is a non-volatile sesquiterpenoid phytoalexin that is effective in plant defense responses against different species of pathogenic fungi, such as *C. sublineolum*, *F. graminearum*, *C. heterostrophus*, *A. flavus*, and *Rhizopus microsporus*. It also has a moderate response in *C. graminicola* (Christensen et al., 2018; Huffaker et al., 2011). Gene silencing of the *tps6* and *tps11* genes, which are involved in the synthesis of zealexins in maize, showed that plants infected with *U. maydis* were more susceptible against fungal infection. This study demonstrated the antimicrobial activity of zealexins in maize (Van Der Linde et al., 2011). Other studies have revealed similar results: during the infection of *F. verticillioides,* TPS6 is elicited in response to pathogens (Veenstra et al., 2019) and is upregulated particularly during the early stages of infection (24 hpi) (Wang et al., 2016b). Furthermore, TPS6 is also expressed during *C. zeina* infection in maize (Meyer et al., 2017).

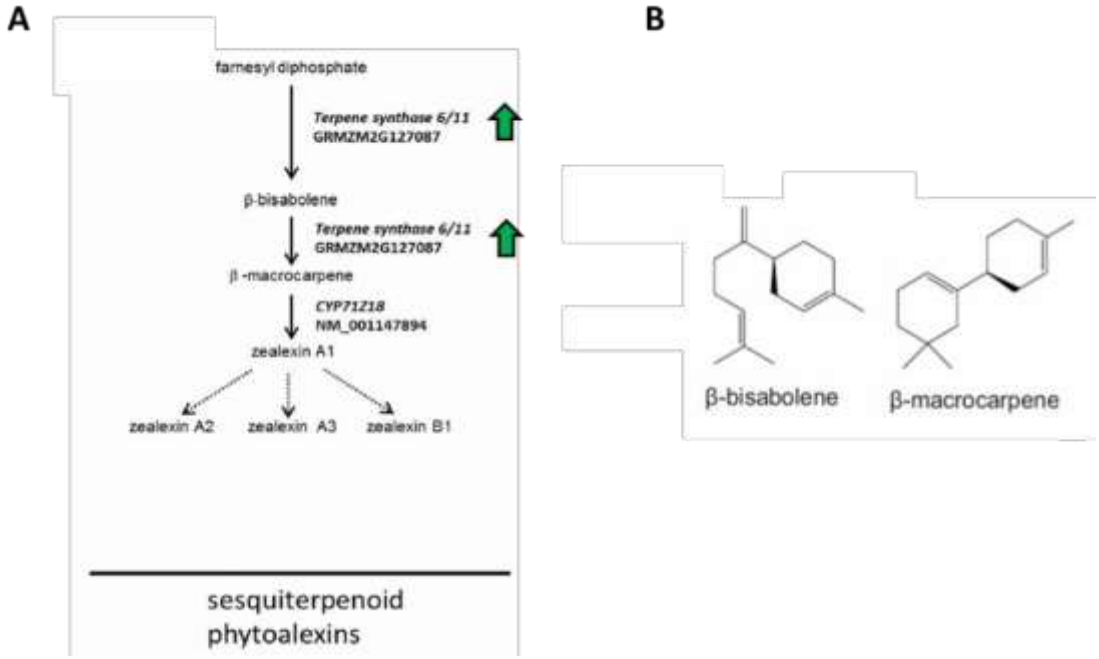


Figure 56. CgEP3 effector could modulate secondary metabolism through sesquiterpenoid phytoalexin pathway. A) Zealexin biosynthesis pathway. The enzymes and reactions connected by the bold and solid arrows have been demonstrated experimentally, while those connected by the dashed arrows represent putative pathways downstream of zealexin A1 synthesis. CgEP3 could induce the expression of TPS6 involved in β-bisabolene and β-macrocarpene which is the precursor of zealexins with antifungal effects. Green arrows with black borders represent the genes induced by CgEP3. This figure has been modified from the original that is found in the article by Meyer et al. (2017). **B)** Molecular structure of β-bisabolene and β-macrocarpene. This figure has been modified from the original that is found in the article by Block et al. (2019).

GENES RELATED TO RNA METABOLISM

DNA directs all the developmental processes sustaining through the intermediate molecules known as RNAs, which are translated to form a wide variety of proteins in the cells. From the formation of an RNA molecule to its degradation, it interacts with numerous proteins. These associations play an important role in various biological processes, such as gene regulation, coding and non-coding RNA production, transport, translation, and RNA removal. It can also participate in various processes related to growth and diseases. RNA-binding proteins (RBPs) are proteins that are related to RNA (Burjoski & Reddy, 2021). Similarly, plant RBPs participate in different general processes, such as growth and development, genome organization, responses to various stresses, plant defense responses, mRNA processing, and post-transcriptional regulation (Burjoski & Reddy, 2021; Dedow & Bailey-Serres, 2019; Huh & Paek, 2013; Köster et al., 2017; Lee & Kang, 2016; Lorković, 2009; Marondedze, 2020; Woloshen et al., 2011). We found several genes related to RNA metabolism that could be affected by CgEP3. These genes are *GRMZM5G865151*, *GRMZM2G421231*, *GRMZM2G137596*, and *GRMZM2G074769*, and were suppressed by CgEP3 **(Supplementary Table 7, Figures 57, and 58)**.

The maize *GRMZM5G865151* gene is annotated to express a putative member of the Ser/Arg-rich (SR) protein family (Goodstein et al., 2012) **(Supplementary Table 7 and Figure 57)**. SR proteins belong to a family of splicing factors that are capable of binding to certain pre-mRNA sequences. These are known as intronic or exonic splicing enhancer or suppressor sequences (Staiger & Brown, 2013; Witten & Ule, 2011). SR proteins also participate in alternative splicing, which, primarily among numerous other functions, plays an important role in plants subjected to environmental stresses. Gene expression can be changed through alternative splicing produced by splicing factors (Staiger & Brown, 2013). One example is the splicing factor Ad-RSZ21 of *Arachis diogoi.* It is an SR protein that is associated with At-RSZ22 from *A. thaliana,* which plays a role in plant defense, inducing a hypersensitive response and consequently, cell death. It also participates in the upregulation of different transcripts implicated in defense (Kumar & Kirti, 2012; Staiger & Brown, 2013). Homologs of this gene in rice (*LOC_Os01g57150.1*) encodes a member of the SR family of proteins that belongs to the QTL (*qTGW1.2a*) and is associated with grain weight and size in rice (Wenhui et al., 2019). In *A. thaliana,* the homologs of this gene (*AT1G61170.1*) encodes an unknown protein. Yan et al. (2016), thanks to yeast two-hybrid experiments, showed that this protein can interact with AtTRN1. It is a transportin that participates in the transport of numerous nucleo-cytoplasmic proteins. Although the protein AT1G61170.1, can link with AtTRN1, its function remains unknown (Yan et al., 2016).

The maize gene *ZmPPR310* (*GRMZM2G421231*) encodes a pentatricopeptide repeat protein 310 (Wei & Han, 2016) **(Supplementary Table 7 and Figure 57)**. Pentatricopeptide repeat proteins (PPR), which are encoded in the nucleus, are a superfamily of proteins that can bind to RNA (Wei & Han, 2016). PPR proteins play an important role in RNA metabolism in chloroplasts and mitochondria, such as endonucleolytic processing, splicing, editing, and translation initiation (Barkan & Small, 2014; Manavski et al., 2018; Shikanai & Fujii, 2013). Additionally, PPR proteins can stabilize mRNAs, rRNAs, and tRNAs (Barkan & Small, 2014; Manavski et al., 2018). Wei & Han (2016) observed several *ZmPPR* genes that were differentially expressed during fungal *U. maydis* infection. Thus, ZmPPRs may be associated with the defense response of maize against pathogens. In the study by Christie et al. (2017), *ZmPPR310* was downregulated when maize was infected with *C. zeina.*

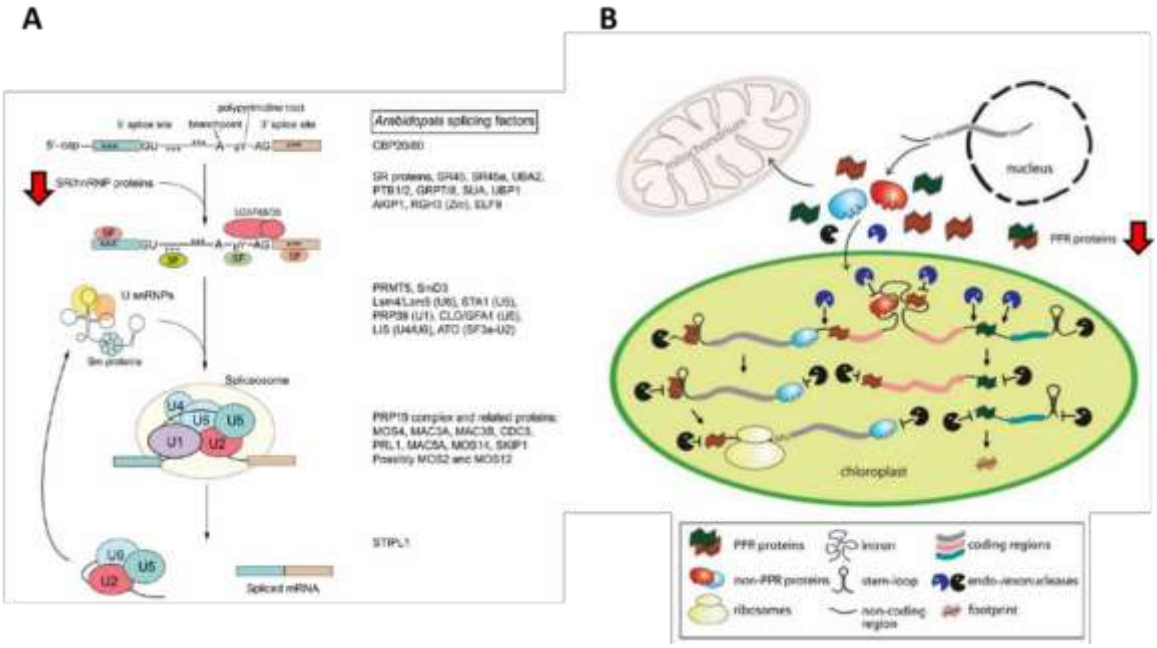


Figure 57. CgEP3 effector could modulate RNA metabolism. A) Model of the components that participate during pre-mRNA processing: splicing signals, factors, and spliceosome. CgEP3 could suppress SR protein. This figure has been modified from the original that is found in the article by Staiger & Brown (2013). **B)** Operational scheme of PPR proteins that are encoded by the nucleus. They protect chloroplast RNAs from the attack of endo or exonucleases. CgEP3 could suppress ZmPPR330 protein. This figure has been modified from the original that is found in the article by Manavski et al. (2018). In both cases, red arrows with black borders represent the genes that are suppressed by CgEP3.

The maize gene *GRMZM2G137596* was annotated as mitochondrial ribosomal protein S34 (Jones et al., 2014; Mitchell et al., 2019) **(Supplementary Table 7 and Figure 58)**. Homologs of this gene in *A. thaliana* encode a mitoribosomal protein, mS34 (Tomal et al., 2019). Mitochondrial ribosomes or mitoribosomes participate in the translation of messenger RNAs (mRNAs) in the mitochondria. Mitoribosomes consist of small and large subunits (SSU and LSU, respectively), which are composed of mitoribosomal RNAs and proteins (mtRPs). The mRNA binds to the SSU, which participates in the interaction of the mRNA with the anticodons of the transfer RNA. Translation of mRNA into proteins occurs (Ban et al., 2020; Tomal et al., 2019). Recent studies have proposed that mitochondrial ribosomes can also help in regulating gene expression. Growth and development processes, such as embryogenesis and the formation of the leaf and reproductive tissues are affected in plants with mutant genes that encode different mtRPs (Robles & Quesada, 2017). *Arabidopsis thaliana* mtRP ms34 belongs to the SSU (Tomal et al., 2019). However, the action of mtRP mS34 during plant development or defense remains unclear.

The maize *GRMZM2G074769* gene was annotated as RP-L10Ae, RPL10A, and large subunit ribosomal protein L10Ae (Kanehisa et al., 2016) **(Supplementary Table 7 and Figure 58)**. Homologs of this gene in rice (*LOC_Os08g44450.1*) encodes an OsL10a putative 60S ribosomal protein L10. This protein is conserved in all organisms, including prokaryotes and eukaryotes. However, its function remains unclear. OsL10a is a putative ribosomal factor in the large subunit complex, and it overlaps with the 60S ribosomal subunit fraction (Im et al., 2011). *LOC_Os08g44450.1* is associated with enhanced bacterial blight resistance in *X. oryzae* pv. *Oryzae* (Jung et al., 2014). Homologs of this gene in *A. thaliana* (*AT1G08360.1*) takes part in *PIGGYBACK* genes, which encode 60S ribosomal proteins L10a, L9, and L5, which affects the

development of leaves and their patterns (Im et al., 2011; Pinon et al., 2008). It has also been shown that this gene is downregulated against *Agrobacterium tumefaciens* infection (Ditt et al., 2006).

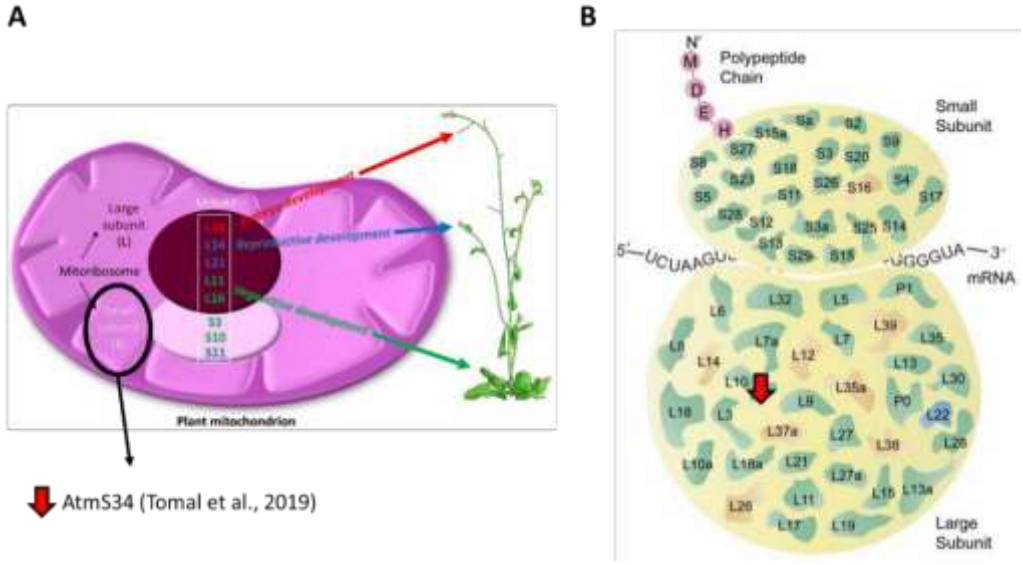


Figure 58. CgEP3 could modulate ribosomal proteins. A) Diagram of mitoribosomal proteins (MitoRPs) which have been deleted in previous studies. The mutation affected plant growth and development. Based in *A. thaliana* studies, CgEP3 could suppress the homologous gene *AtmS34* of maize. This figure has been modified from the original that is found in the article by Robles & Quesada (2017). **B)** Scheme of *A. thaliana* ribosome. The ribosomes are composed of ribonucleoprotein complexes. They participate in the translation of proteins. *Arabidopsis thaliana* has 80 types of ribosomal proteins. CgEP3 could suppress subunit L10A of ribosome. This figure has been modified from the original that is found in the article by Wang et al. (2013). In both cases, red arrows with black borders represent the genes that are suppressed by CgEP3.

GENES RELATED TO PROTEIN FOLDING AND DEGRADATION

Protein activity can be modulated through post-translational modifications that are important for protein stability. Different types have been described, including phosphorylation, methylation, acetylation, myristoylation, glycosylation, and ubiquitination. Due to ubiquitination, proteins are derived through the degradation pathway by the 26S proteasome. This system constitutes the most important pathway for protein degradation in cells. Ubiquitination can also participate in the control of numerous eukaryotic cell processes. A wide variety of E3 ubiquitin ligases, their substrates, and other critical cellular regulators have been identified (Zeng et al., 2006). The ubiquitination system in plants is vital for numerous growth and development processes, as well as responses against biotic (against pathogens) and abiotic (drought, salinity, cold, and nutrient starvation) stresses (F. Q. Xu & Xue, 2019; Zeng et al., 2006). We found several genes related to protein folding and degradation that could be affected by CgEP3. The genes *GRMZM2G384706*, *GRMZM2G449083*, *GRMZM2G057374,* and *GRMZM2G132811*, were suppressed by CgEP3, and *GRMZM2G090689* was induced by CgEP3 **(Supplementary Table 7, Figures 59, and 60)**.

The maize gene *GRMZM2G384706* is annotated as a U-box domain-containing protein (Goodstein et al., 2012) **(Supplementary Table 7 and Figure 59)**. Homologs of this gene in rice (*LOC_Os03g13010*) encodes a putative U-box E3 ubiquitin ligase (Ren et al., 2014). The U-box domain is considered to play a role in ubiquitination (Aravind & Koonin, 2000). U-box proteins

can have a dual function – in protein folding by molecular chaperones, or in degradation via the ubiquitination system (Hatakeyama & Nakayama, 2003). They can also play a role in hormonal responses and biotic stresses (Amador et al., 2001; Ren et al., 2014; Zeng et al., 2004). Some U-box proteins are known to be involved in the defense response of plants against pathogens(González-Lamothe et al., 2006; Kirsch et al., 2001; Ren et al., 2014; C. W. Yang et al., 2006; Zeng et al., 2004). (Zeng et al., 2008), found some U-box genes that could participate in the defense response of rice against the fungal pathogen *M. oryzae.* They could be associated with the regulation of responses and signaling.

The uncharacterized protein encoded by maize gene *GRMZM2G449083* predicted that its structure has LysH-CTLH-CRA-RING-finger domains. The RING domain is a Zn-finger that is involved in protein-protein interactions. It is also implicated in biological processes, such as E3 ubiquitin protein ligase activity, where its function is to determine the specific substrate for ubiquitination (Jaureguibeitia, 2015) **(Supplementary Table 7 and Figure 59)**. Homologs of this gene in rice (*LOC_Os06g38940.1*) is downregulated in the roots when infected with the nematode *Meloidogyne graminicola* (Xiang et al., 2020). Homologs gene in *Arabidopsis* (*AT4G37880.1*) encode the RING/U-box protein, which contains a CTLH-domain whose function is unknown. It is related to AtRanBPM, a plant homologs of human RanBPM. This protein participates in numerous cellular processes through protein folding. Protein AtRanBPM was similar to CTLH types that had been previously characterized in budding yeast and mammals. Although it is still unknown how CTLH complexes function during protein degradation, interaction, signaling, etc., the preservation of these complexes in eukaryotic organisms shows that their function is essential (Tomaštíková et al., 2012).

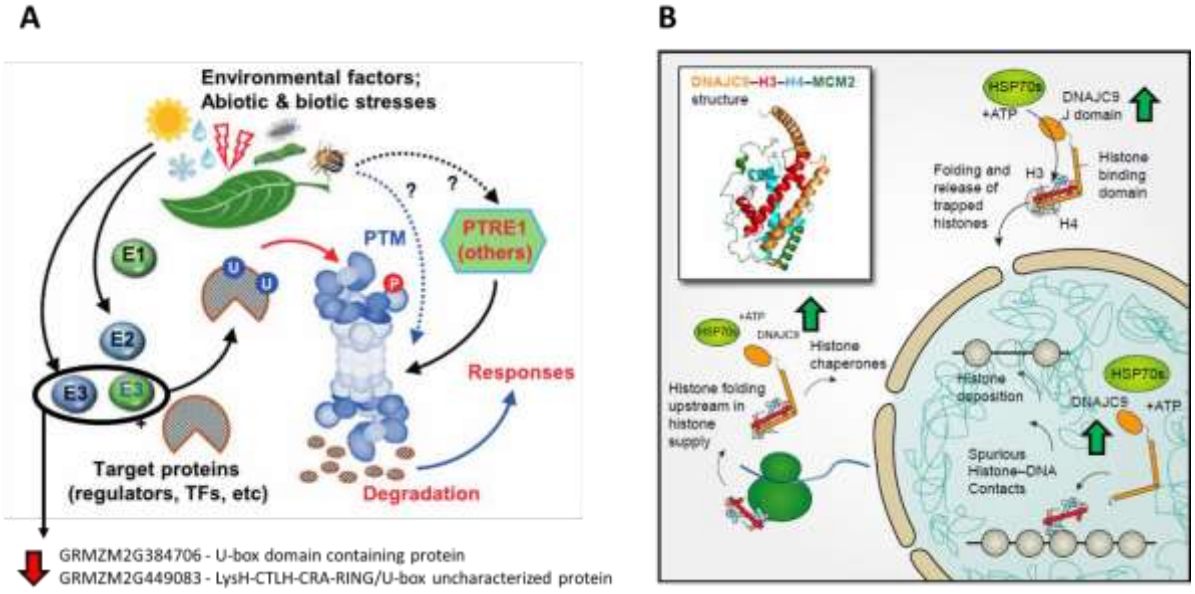


Figure 59. CgEP3 could modulate protein folding and degradation. A) Model of the ubiquitination and degradation system via proteasome of plants that are responsive to stress. This system specifically degrades proteins encoded by genes which have been previously induced by stress. This allows the degradation of the target protein resulting in defense response. CgEP3 could suppress these hypothetical U-box E3 ubiquitin ligases (GRMZM2G384706 and GRMZM2G449083). This figure has been modified from the original that is found in the article by Xu & Xue (2019). **B)** Based on the human model, DNAJC9 acts as histone chaperone and heat-shock co-chaperone. DNAJC9 participates in folding and release of trapped histones. CgEP3 could induce the homologs gene *DNAJC9* in maize. This figure has been modified from the original that is found in the article by Hammond et al. (2021). In both cases, red and green arrows with black borders represent the genes that are suppressed or induced respectively by CgEP3.

The maize gene *ZmFKBP17-3* (*GRMZM2G057374*) is annotated as FK506-binding protein (FBKP) and its predicted localization is the thylakoid lumen of chloroplasts (Y. Yu et al., 2012) **(Supplementary Table 7)**. This protein belongs to the peptidyl-prolyl cis/trans isomerase superfamily, which plays a role in various processes, such as protein folding, hormone signaling, plant growth and development, and stress responses (Y. Yu et al., 2012). Ding et al.(2019), found that this gene is upregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. Homologs of this gene in *A. thaliana* (*AT1G18170.1*) encodes a FKBP17-2 protein. It has appeared in the co-expressed gene network of the thylakoid lumen of chloroplasts (Ifuku et al., 2010) and is involved in protein folding (Kleine, 2012). In a study by Fabro et al. (2008), this gene was reported to be downregulated during the early interaction of the obligate biotrophic fungus *Golovinomyces cichorancearum* in *A. thaliana.*

The maize gene *ZmCYP33* (*GRMZM2G132811*) encodes a cyclophilin protein (CYP) (Wang et al., 2017). Although previous reports have designated a mitochondrial localization for the protein, analyses from this study suggests a subcellular localization of the protein in the chloroplasts **(Supplementary Table 7)**. Cyclophilin proteins belong to the peptidyl prolyl cis/trans isomerases. These enzymes can act during the cis/trans isomerization of amino acid proline (Gan et al., 2009; Gething, 1997; Wang et al., 2017). CYPs are molecular chaperones, which are involved in the proper folding of proteins, assembly, and stabilization (Hartl et al., 1994; Trivedi et al., 2012; Qianqian Wang et al., 2017). Additionally, CYPs also play a role in other cellular processes, such as cell signaling, cell division, apoptosis, transcriptional regulation, RNA processing, spliceosome assembly, and response to biotic and abiotic stresses (Dominguez-Solis et al., 2008; Wang et al., 2017). Ding et al. (2019), found that this gene is downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. There are other examples of cyclophilin proteins in *A. thaliana* that participate in microbe interactions, such as *AtCYP19-1* and *AtCYP57.* These genes were activated against *P. syringae* and *X. campestris* bacterial infections. Thus, cyclophilins can participate in plant defense responses against these species of bacteria (Barbosa dos Santos & Park, 2019; Mokryakova et al., 2014; Pogorelko et al., 2014).

The maize *GRMZM2G090689* gene was annotated as DnaJ homolog subfamily C member 9 (DNAJC9) (Kanehisa et al., 2016) **(Supplementary Table 7, Figure 59)**. Homolog of this gene in rice (*LOC_Os02g10220.1*) is described as OsDjC17 (Sarkar et al., 2013). J-proteins/DnaJ proteins participate in basic cellular processes, development, and stress. They are involved in protein folding and disaggregation, which are important under adverse cell conditions. The molecular chaperones, heat shock proteins (Hsp70 and Hsp100) form an important network that assists in these processes. J-proteins/DnaJ and molecular co-chaperones help Hsp70 (Sarkar et al., 2013). Homologs of this gene in *A. thaliana* (*AT3G12170.1*) encodes a chaperone from the DnaJ-domain superfamily of proteins (Goodstein et al., 2012). It has been shown that this gene is strongly associated with the human nuclear H3-binding factor DNAJC9 (E. I. Campos et al., 2015; Z. Hu et al., 2019; Lambert et al., 2015). The entrance to DNA varies depending on the position and composition of nucleosomes. This fact influences numerous characteristics of genomic function, such as gene expression, silencing of repetitive elements, and DNA repair (Allis & Jenuwein, 2016; Hammond et al., 2021; W. K. M. Lai & Pugh, 2017; Nicetto & Zaret, 2019; T. Yadav et al., 2018). Thus, to ensure suitable contact of nucleosomes during assembly, histone chaperones buffer the interplay between histones and DNA (A. J. Andrews et al., 2010; Hammond et al., 2021). In *A. thaliana,* the gene *AT3G12170.1* is associated with the brassinosteroid (BR) signaling pathway. It forms part of a group of genes that are downregulated in plants that constitutively activate BR. The BR hormonal pathway plays a key role in several processes, such as development, response to biotic and abiotic stresses (H. Kim et al., 2016) .

GENES RELATED TO PLANT CELL REMODELING

The plant cell wall is structurally one of the most complex networks on Earth. It consists of a great diversity of biological polymers, and numerous genes are involved in its construction (B. Zhang et al., 2021). The cell wall mechanically supports the plant and fulfills an important function during growth and development, protecting it against unfavorable conditions, such as biotic and abiotic stresses. The structure and composition of the cell wall are constantly changing in response to the specific needs of the plant as well as diverse biological processes. The mechanism of maintenance of plant cell wall integrity (CWI) helps plants support their integrity during the growth of cell walls and when interacting with the environment and pathogens (Gigli-Bisceglia et al., 2020). After a mechanical or biochemical damage to the cell wall by the pathogen, the cell starts a wall repair process that avoids the spread of infection. The CWI maintenance mechanism produces an adaptive response against damage in the cells, and in turn, the basal defense response PTI is triggered in the plant (Engelsdorf et al., 2018). We found one gene related to cell wall remodeling that could be affected by CgEP3. This gene is *GRMZM2G000976* **(Supplementary Table 7)**.

The maize gene *GRMZM2G000976* belongs to the glycosyltransferase 47 family (GT47) and is a carbohydrate-active enzyme (CAZy). It is predicted that this protein is related to xyloglucan biosynthesis through the enzyme xylogalacturonan β-1,3-xylosyltransferase (Ekstrom et al., 2014). Homologs of this gene in *A. thaliana* (*AT4G38040.1*) encodes CAZy GT47 (Geshi et al., 2018). GT47 is a family of enzymes composed of glycosyltransferases. Predicting the function of these enzymes is complicated because they are associated with different donors and acceptors, and consequently, biochemical characterization is necessary. Many of the GT47 proteins participate in plant cell wall biosynthesis and are found in vesicles of the Golgi apparatus. The in-silico expression of the single-copy gene *AT4G38040.1* was strong and ubiquitous. However, deletion of this gene is necessary to determine its function (Geshi et al., 2018). GT47 family proteins have been studied in other plant hosts, such as barley (J. Chowdhury et al., 2017). They proposed that GT47 is a candidate gene that enhances resistance against *Blumeria graminis* f. sp. *hordei,* which causes powdery mildew disease in barley leaves. It is co-expressed with the GT43 gene, and both could play an important role in the synthesis of the xylan backbone. Papillae deposition occurs during powdery mildew infection. The heteroxylan of papillae could contribute to the resistance of the plant against penetration by the fungus. Diverse GT families are involved in plant cell wall heteroxylan assembly. GT families can work together to form a multi-enzyme complex. During mildew infection in barley, heteroxylan biosynthesis genes are upregulated in the leaf epidermis. Thus, these genes could contribute to the resistance of the fungal penetration through changes in the abundance and structure of the heteroxylans in the papillae depositions (J. Chowdhury et al., 2017). Although there is an example of GT47 proteins that could be involved in plant-pathogen interaction, the function of the GT47 maize protein (GRMZM2G000976) remains unknown.

OTHER GENES

There are other genes that could be suppressed or induced by CgEP3, which have not been classified in any category. Among them *GRMZM2G095634*, *GRMZM2G407913*, *GRMZM2G147014*, and *GRMZM2G035636* are suppressed by CgEP3, while *GRMZM2G085711*; *GRMZM2G045675*, *GRMZM2G090245* and *GRMZM5G836353* are induced by CgEP3 **(Supplementary Table 7)**.

Genes that are suppressed by CgEP3

The serine/threonine protein casein kinase I (CK1) is found in all eukaryotic organisms. It participates in numerous important cellular processes, such as metabolism, developmental monitoring, cell cycle progression, and stress response (Kang & Wang, 2020). The maize gene *GRMZM2G095634* is annotated as CK1, kinase (Kanehisa et al., 2016) **(Supplementary Table 7)**. Ding et al. (2019) found that this gene is upregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. He et al. (2016), reported that it is overexpressed in roots suffering from nitrogen deficiency. It functions as part of a group of genes that regulate the nitrogen deficiency responsive network.

The maize gene *GRMZM2G407913* was annotated as protein SEY1 (Kanehisa et al., 2016) **(Supplementary Table 7)**. The thesis dissertation of (Grégoire, 2013), reported homologs of this gene in rice (*LOC_Os12g41170.1*) to be downregulated in creeping bentgrass plants (*Agrostis stolonifera*) when infected with *Sclerotinia homeocarpa,* which produces the dollar spot disease. Heterologous microarray hybridization using a rice oligonucleotide array was analyzed to measure bentgrass gene expression during infection with *S. homeocarpa* (Grégoire, 2013). Homologs of this gene in *A. thaliana* (*AT5G45160.1*) encodes a RHD3-like 2 protein that may control, together with RHD3, the tubule fusion in the endoplasmic reticulum (ER) (Stefano et al., 2014). RHD3-like 2 is expressed ubiquitously at very low levels in plant tissues (Chen et al., 2011; Hu et al., 2003; Schmid et al., 2005).

Maize gene *DEH2* (*GRMZM2G147014*) encodes a dehydrin (DEH) protein (Engelberth et al., 2019) **(Supplementary Table 7 and Figure 43)**. DEHs are a group of protein that are normally activated under certain stresses, such as drought and cold (Borovskii et al., 2002; Engelberth et al., 2019; Rorat, 2006). DEHs have a lysine-rich K-segment in their structure, which is ubiquitous in plants. DEHs are found in all cell types and are involved in stabilizing membranes and proteins in the cell compartments where they are found (Borovskii et al., 2002; Engelberth et al., 2019; Imamura et al., 2013; Koag et al., 2003; Szabala et al., 2014; Vaseva et al., 2014). Ding et al. (2019) found that this gene is downregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. In the thesis dissertation of Korsman (2015), this gene has stronger expression in susceptible bulked recombinant inbred maize lines during *C. zeina* infection. It is related to a QTL (5Cz) that could control the growth of *C. zeina* within leaves; thereby reflecting the pathogen content in leaves. Homologous genes in *A. thaliana,* dehydrin *COR47* (*AT1G20440.1*), decreased in abundance during *P. syringae* pv. *tomato* DC3000. Hydric stress limits bacterial growth. *Arabidopsis thaliana* plants infected with *P. syringae* pv. *tomato* DC3000 at 4 hpi reduced their water potential (Borovskii et al., 2002; Jones et al., 2006). Thus, dehydrins function by controlling the hydric relationship between the host and the pathogen (Jones et al., 2006).

The maize gene *GRMZM2G035636* is annotated as phytepsin, which is related to the aspartic peptidase family of proteins (Kanehisa et al., 2016) **(Supplementary Table 7)**. The aspartic protease (AP) family is one of the most important families of proteases. In plants, they can participate in numerous processes, such as defense against biotic and abiotic stresses, the processing and degradation of proteins, programmed cell death, and senescence (Y. Yang & Feng, 2020). Homologs of this gene in rice (*LOC_Os05g49200.1*) is induced 24 h after infestation of the Asian rice gall midge (*Orseolia oryzae*) (Divya et al., 2016). The protein encoded by this gene has been identified in rice leaves during both early infection (Buffon, Blasi, Adamski, Ferla, Berger, Santi, Lavallée-Adam, Yates, et al., 2016) and late-infestation by the mite *Schizotetranychus oryzae* (Blasi et al., 2017). Niehl et al. (2013), presented that homologs of this

gene in *A. thaliana* (*AT1G11910.1*) induced accumulation against the Oilseed rape mosaic virus. Other studies on AP have shown its role in plant resistance against pathogens, such as AP nephenthesin in wheat. It is highly upregulated in *B. graminis* f. sp. *tritici,* which causes wheat powdery mildew disease (Yang & Feng, 2020), and other hosts, including tomato, tobacco, potato, *Cucurbita maxima,* rice, and *A. thaliana* (Alam et al., 2014; F Rocha et al., 2015; Frey et al., 2018; Guevara et al., 2001; Mendieta et al., 2006; Rodrigo et al., 1989; Sebastián et al., 2020).

Genes that are induced by CgEP3

In maize, *GRMZM2G085711* encodes an apyrase protein (Kanehisa et al., 2016) **(Supplementary Table 7)**, which is associated with the hydrolysis of nucleotide triphosphate to nucleotide monophosphate in two different successive phosphate release steps, with diphosphate nucleotides as intermediates (Portwood et al., 2019). Shi et al. (2018) reported that the gene *GRMZM2G085711*, putatively encoding apyrase family protein, was downregulated in response to infection by the hemibiotrophic fungus *S. turcica* at 60 hpi. Homologs of this gene in *A. thaliana* (*AT5G18280.1*) encodes the apyrase AtAPY2 (J. Choi et al., 2014). Extracellular ATP, which is hydrolyzed by the action of apyrases, plays an important role in defense responses of the plant (J. Choi et al., 2014). When the *AtAPY1* and *AtAPY2* genes were suppressed, extracellular ATP started to increase. Thus, plants express numerous genes related to stress (J. Choi et al., 2014; M. H. Lim et al., 2014). Some studies support the function of apyrases during biotic stress in plants (Cao, Tanaka, et al., 2014; Clark et al., 2014). The apyrase PsAPY1 participates in pea immunity by binding PsAPY1 with both defense elicitor and a suppressor isolated from the pathogenic fungus *Mycosphaerella pinoides* (J. Choi et al., 2014; Kiba et al., 2006). Tobacco plants that constitutively expressed the *PsAPY1* gene showed resistance against *Alternaria* sp. and *P. syringae* pv. *tabaci* (J. Choi et al., 2014; Shiraishi, 2013).

The maize gene *GRMZM2G045675* is annotated as a putative dehydration response-related protein (Goodstein et al., 2012) **(Supplementary Table 7)**. Homologs of this gene in *A. thaliana* (*AT4G00750.1*) is a dehydration-responsive gene that contains activation sequence-1 (as-1) elements in its promoter (Rama Devi et al., 2006). Genes that have as-1 elements in their promoter regions are associated with plant defense responses. The as-1 elements can help activate these genes and are able to respond to numerous stresses, such as silicic acid, jasmonic acid, and H_2O_2, among others (Rama Devi et al., 2006). In a study by (Postnikova & Nemchinov, 2012), this gene responds to several viral infections in *A. thaliana* plants. They analyzed microarray data from 11 species of viruses that infect *A. thaliana.*

Germin (GERs) and germin-like proteins (GLPs) participate in the superfamily of "cupin" proteins. These proteins can participate in important processes in the plant, such as growth, development, and defense. GERs can be transported from the rupture of oxalate (oxalic acid), the products of which are H_2O_2 and CO_2 (Ilyas et al., 2016). The functions of numerous GERs and GLPs in different plant species have been described. They are exposed to several biotic and abiotic stresses, especially against fungal pathogens. These studies demonstrated the importance of these proteins in developmental, biochemical, and physiological processes. (Davidson et al., 2010; Dunwell et al., 2008; Ilyas et al., 2016; Manosalva et al., 2009). The maize gene *GRMZM2G090245* was annotated as germin-like protein subfamily 3 member 3 (PTHR31238) (Mi et al., 2013; P. D. Thomas et al., 2003) **(Supplementary Table 7)**. The thesis dissertation of Shu (2014) reported the up-regulation of this gene against *A. flavus* and *F. verticillioides* infection in maize plants. In the study by Yuan et al. (2018), homologs of this gene in rice, *LOC_Os08g35750.1* is reportedly expressed against the fungus *R. solani,* which causes

rice sheath blight. They suggested that this extracellular gene together with others can activate the immune response in the rice resistance cultivar YSBR1 after 6 hpi. Similarly, homologs of this gene in *A. thaliana, AT1G72610.1,* is an extracellular apoplastic protein that is predicted to be related to the category of disease/defense responses (Takahashi et al., 2016). In a study by (Mulema & Denby, 2012), this gene was downregulated when *A. thaliana* plants were inoculated with the gray mold fungus *B. cinerea* at 24 hpi.

The maize gene *GRMZM5G836353* is predicted to encode monooxygenase 1 (Shi et al., 2019) **(Supplementary Table 7)**. According to the literature consulted on maize and *A. thaliana*, this gene could participate in different pathways. Shi et al. (2019) reported that *GRMZM5G836353,* together with other genes of the chlorophyll biosynthesis pathway could take part in QTLs that are implicated in chlorophyll content. Ding et al. (2019), found that this gene is upregulated in maize infected with the hemibiotrophic fungus *C. heterostrophus* at 24 hpi. Homologs of this gene in *A. thaliana* (*AT4G15760.1*) encodes monooxygenase/aromatic-ring hydroxylase I (MOI), which is upregulated in the presence of the necrotrophic fungus *Alternaria brassicicola* (Mukherjee et al., 2009). Li et al. (2014), discovered that this gene was upregulated in the presence of the phytohormone strigolactone. This molecule can regulate plant development and stress responses. In contrast, Ramel et al. (2012), suggested that this gene might be involved in detoxification processes.

UNKNOWN GENES

Finally, there are several genes on which little or no information was readily available in either the digital databases or existing literature. Among these genes, *GRMZM2G145563*, *GRMZM2G164963*, *GRMZM2G092758*, *GRMZM2G517065*, *GRMZM2G153635* and *GRMZM2G052255*, were suppressed by CgEP3, while *GRMZM2G029810*, *GRMZM5G813709* and *GRMZM2G056216* were induced by CgEP3 **(Supplementary Table 7)**.

The unknown gene *GRMZM2G145563* **(Supplementary Table 7)** appears to be upregulated in the maize resistant line RIL387 during *C. zeina* leaf infection (Meyer et al., 2017). This could also be related to maize kernel weight, because this gene belongs to a group of QTLs that are associated with kernel weight, besides being important for maize domestication (Shannon, 2013).

GRMZM2G164963 in maize encodes an uncharacterized protein (Jones et al., 2014; Mitchell et al., 2019) **(Supplementary Table 7)**. Huang et al. (2013), reported homologs of this gene in rice (*LOC_Os04g31010.1*) to be related to the locus of the resistant gene *BPH27* (86.3-kb region) against brown plant hopper (*N. lugens*). They identified six other resistant genes, together with *BPH27.* However, the function of *LOC_Os04g31010.1* is unknown. Following the PlantSPR database (Tian et al., 2009), it was predicted that its subcellular localization could be the chloroplast. The thesis dissertation of Plohmann, (2015) proved that homologs of this gene in *A. thaliana* (*AT4G22830.2*) is co-expressed with *VTE6* (phytyl phosphate kinase), which plays a role in tocopherol biosynthesis in chloroplasts. Tocopherols are fat-soluble antioxidant compounds that participate in the defense of plants against biotic and abiotic stresses (Cela et al., 2018a; Munné-Bosch, 2005; Phillips et al., 2020) . Tocopherols help decrease the concentration of ROS in the photosynthetic apparatus. They also defend photosynthetic membranes from lipid peroxidation, and consequently, the membranes remain stable against environmental stresses (Munné-Bosch et al., 2013; Munné-Bosch & Alegre, 2002; Zaid & Wani, 2019)

DISCUSSION

RNA-Seq studies allow us to comprehensively analyze the responses that occur during host-pathogen interactions. Thus, transcriptome profiling helps us understand the development of the disease caused by pathogens and the resistance of the host during the infection process (Bhat et al., 2019a; Kawahara et al., 2012). Here, we studied interactions within the *C. graminicola*-maize pathosystem and specifically, the function of CgEP3, which is secreted by the fungus to counteract defenses and enhance colonization within the host. We found 56 DEGs when maize leaves were inoculated with Δ*CgEP3* compared to WT treated leaves during the early stage of infection (24 hpi). We have referred to them as effector-responsive genes, and they are regulated in the absence of an effector. Among them, 35 DEGs were upregulated, and they could be suppressed in the presence of CgEP3, and 21 DEGs were downregulated in the presence of CgEP3. We have seen that numerous pathways are important for plant immunity and many of them are affected by CgEP3; however, it is unknown whether the action of CgEP3 in these genes is directly or indirectly affected.

Pathogenesis-related proteins (PR) have been shown to participate in the fight against pathogenic infections, and some of them have direct antimicrobial activity against pathogens (van Loon et al., 1994; Van Loon et al., 2006; Zhi-ming et al., 2021). Among these genes, CgEP3 induced the expression of *PRB1-3-like* and *PR2*. Maize can recognize CgEP3 and induce its expression. PR proteins are induced during the PTI defense response to fight pathogen invasion (de Jesus Miranda et al., 2017). Several studies have shown that these proteins are vital in improving maize defense responses during *C. graminicola* infection (Balmer et al., 2013; Bhat et al., 2019b; Miranda et al., 2017; Vargas et al., 2012). PRB1-3-like possesses lysM domains in its structure, which could be linked with chitin elicitor binding protein (Bhat et al., 2019). Members of the glucanase superfamily, such as β-1,3-glucanase (PR-2), can alter the integrity of the hyphal cell wall. Thus, elicitor fragments of β-1,3-glucan are produced, which can trigger the defense response in maize. β-1,3-glucan is necessary for the rigidity of the appressorial cell wall of *C. graminicola* (Miranda et al., 2017; Oliveira-Garcia & Deising, 2013).

Knowledge of the maize response mechanism against *C. graminicola* related to phytohormones is very limited. Previous studies have shown that SA, ABA, and JA may play a role in defense (Balmer et al., 2013; Bhat et al., 2019a; De Jesus Miranda et al., 2017; Gorman et al., 2020; Vargas et al., 2012). Generally, infection with *C. graminicola* can trigger a defense response that depends on the SA (Balmer et al., 2013). Because PR-related genes are induced by CgEP3, we speculated that the SA pathway was activated during early infection by *C. graminicola*. SA has been widely examined because it actively participates in the local and acquired responses during infection by biotrophic and hemibiotrophic pathogens (Dempsey et al., 2011; Xiaowei Han & Kahmann, 2019) ABA is a fundamental hormone that contributes to the growth and development of plants, as well as adaptation to various biotic and abiotic stresses (Cutler et al., 2010; Han & Kahmann, 2019). However, ABA negatively affects the resistance of plants against biotrophic and hemibiotrophic pathogenic filamentous fungi, such as *Hyaloperonospora arabidopsidis*, *F. graminearum*, *M. oryzae*, and *G. cichoracearum*(Buhrow et al., 2016; Fan et al., 2009; Xiaowei Han & Kahmann, 2019; Jiang et al., 2010; X. Yu et al., 2017). In contrast, ABA is important for plant resistance to necrotrophic pathogens such as *Pythium irregulare*, *P. cucumerina*, *C. miyabeanus*, and *A. brassicicola* (Adie et al., 2007; de Vleesschauwer et al., 2010; Fan et al., 2009; Xiaowei Han & Kahmann, 2019; Ton & Mauch-Mani, 2004). CgEP3 could suppress the NCED8 enzyme, which participates in the first step of ABA biosynthesis (Dutta et al., 2019a; Schwartz et al., 1997), and this pathway may be affected. In a previous study, the

application of ABA to maize plants infected with *C. graminicola* increased the susceptibility of plants to the pathogen (Vargas et al., 2012). In contrast, Balmer et al. (2013), reported increased resistance to ABA treatment. This could be the result of changes in ABA function during disease development, which has been observed in other host-pathogen interactions (Ton et al., 2009). ABA promotes plant resistance when ABA treatment is carried out during the early stages of maize anthracnose. However, when the pathogen penetrates the plant epidermis in later stages, ABA can affect the generation of ROS, thus causing an increase in susceptibility (Balmer et al., 2013). Thus, CgEP3 might suppress the ABA pathway during the early stages of infection and improve maize susceptibility to *C. graminicola.* On the other hand, CgEP3 could induce the expression of genes related to the ET signaling pathway (EIN2-like) and GA biosynthesis pathway (cytochrome P450, *A. thaliana* homologous CYP72A15). ET signaling can also be involved in defense responses. In an experiment carried out with *A. thaliana* and soybean plants that were insensitive to ET, they were shown to be susceptible to certain pathogens, but activation of the ET signaling pathway improved resistance to pathogen aggression (Berrocal-Lobo et al., 2002; Xiaowei Han & Kahmann, 2019; Hoffman et al., 1999; Thomma et al., 1999; C. Yang et al., 2017). Thus, maize plants could detect CgEP3 and improve plant defense by inducing the ET signaling pathway. With respect to GAs, it has been shown that in interaction between rice and the fungus *M. oryzae*. Mutants that participate in the synthesis of GAs, such as gibberellin 20-oxidase (GA20OX3) showed an increase in resistance to rice blast disease (Xiaowei Han & Kahmann, 2019; X. Qin et al., 2013). It was observed that CgEP3 induces the expression of a protein that could be involved in GA biosynthesis. However, the function of GAs during the *C. graminicola*-maize interaction is still unknown.

Control of gene expression is important for the generation of specific genes (Cui et al., 2016). Transcription factors (TFs) regulate diverse processes of plant growth and development, as well as biotic and abiotic stresses (Ng et al., 2018). CgEP3 could affect the expression of five TFs, such as *ZmMYBR115, ZmCOL16, GT* (trihelix), *ZmWRKY83,* and *ZmTPL6.* Plants have 58 different families of TFs (J. Jin et al., 2017), and among them, the MYB and WRKY families are strongly associated with the defense signaling response. Furthermore, both families have functions that overlap in the PTI and ETI signaling cascades (Ng et al., 2018). Therefore, CgEP3 could suppress *ZmMYBR115,* homologous to *A. thaliana,* in resisting *P. parasitica* (Federspiel et al., 2010); and *ZmCOL16,* which is downregulated after maize infection by *C. heterostrophus* (24 hpi) (Y. Ding et al., 2019). Both genes may be important for plant defense, and CgEP3 could suppress them to promote anthracnose development. On the other hand, CgEP3 induced *GT* (trihelix), *ZmWRKY83,* and *ZmTPL6.* It has been observed that genes related to these families are vital in imparting immunity to other hosts, such as *TuGTγ-3* in *Triticum urartu* against stripe rust (Ding et al., 2016); the homologous gene of *ZmWRKY83* in rice against *M. oryzae* (Wei et al., 2013) and *A. thaliana* against *P. syringae* (Yanru Hu et al., 2012); and *ZmTPL6* (*ZmFBX154.1*), which is stimulated during *U. maydis* infection (Villajuana-Bonequi et al., 2019) and homologous in rice, which is expressed against *X. oryzae* pv. *oryzae* at 24 hpi (Kou et al., 2009). These genes are induced after recognition of CgEP3 in maize and may reflect their role in plant defense.

Pathogen attacks increase the requirement for photosynthesis in their hosts. Photosynthesis produces energy and precursor molecules that are necessary for defense responses (Hammerschmidt, 1999; Swarbrick et al., 2006; Zhi-ming et al., 2021). Several studies have shown that pathogens frequently damage and disturb the photosynthetic apparatus of their host plants, particularly the photosystem II complex, to foster the pathogenic process (de Torres Zabala et al., 2015; Ghosh et al., 2017; Nomura et al., 2012; Serrano et al., 2016; Zhi-ming et al., 2021b; Zou et al., 2005). It has been hypothesized that the down-regulation of genes related to

photosynthesis occurs in response to the attack of pathogens during the first hours or days of infection (Bilgin et al., 2010; Bryksová et al., 2020). In this way, CgEP3 can suppress different genes related to photosynthesis, such as DXS, which belongs to the MEP pathway and biosynthesis of plant isoprenoids (photosynthesis pigments, plant hormones and metabolites), which are important in plant defense in different plant pathosystems ((Agudelo-Romero et al., 2008; Y. Ding et al., 2019; Grand et al., 2012; J. Y. Kim et al., 2017; Mitra et al., 2021; Venu et al., 2010). CgEP3 could also affect chlorophyll biosynthesis by suppressing two important enzymes in the process: CHLD and ZmPORA. Both genes are downregulated in *C. heterostrophus* maize infection at 24 hpi (Y. Ding et al., 2019); CHLD against *C. zeina* (Christie et al., 2017) and ZmPORA against *U. maydis* infections in maize (Kretschmer et al., 2017). Thus, the electron transfer chain through the photosystem is affected by the CgEP3 effector. It might suppress chlorophyll a/b-binding proteins of photosystem II and I: ZmLHCBa and LHCA1, respectively. These genes are important for the photosynthetic apparatus of the plant and can be altered by different host-pathogen interactions (Molho et al., 2021; Song et al., 2020; Zhi-ming et al., 2021). Likewise, there is another gene that participates in the electron transfer chain that could be induced by CgEP3, *NdhB* of the NDH-1 complex. Homologs of this gene in *A. thaliana* are upregulated against herbivore attacks (Mata-Pérez et al., 2015). This fact can help plants derive their resources towards defense responses among others (Halitschke et al., 2001; Hui et al., 2003; Mata-Pérez et al., 2015). Maize plants can detect CgEP3 and induce this gene to improve plant defenses. Finally, CgEP3 may suppress *the GRMZM2G070847* gene. Based on its homologs in *A. thaliana,* this gene encodes a protein kinase that is important in starch biosynthesis (Han, 2017). This may be associated with AtCYP38, which can help in photosystem II early biogenesis, correct assembly, and maintenance (Sirpiö et al., 2008). *GRMZM2G070847* was also downregulated in *C. heterostrophus* maize infection at 24 hpi (Y. Ding et al., 2019). Taken together, these results suggest that CgEP3 may suppress photosynthetic components to promote pathogenesis and improve colonization of the host during *C. graminicola* infection.

Plants interact constantly with the environment and microorganisms, and consequently, have developed complex mechanisms to identify them and trigger appropriate defense responses against the eventual pathogens. Essential processes for plant growth and development are downregulated during interactions with pathogens, such as photosynthesis and primary metabolism. The energy that plants conserve from these processes can be utilized to plant the immune response. However, due to diverse studies, primary metabolism is also upregulated during pathogen infection. Thus, primary metabolism can control different signal transduction cascades that allow plant defense responses (Rojas et al., 2014). Thus, CgEP3 could induce or suppress several genes involved in sulfur, selenium, carbon, lipid, and iron metabolism. In sulfur metabolism, CgEP3 could induce the expression of the sulfur transporter *ZmSULTR1.2* and *ATP sulfurylase*, which could be related to plant defense in different hosts and pathogens (Hoopes et al., 2019; Whitley, 2019; Yixiang Zhang, 2014). Sulfur has been implicated in the S-induced defense phenomenon. This is activated when plants respond to pathogens. The principal consequence is the increase in sulfur compounds, which can help alleviate the stress. For example, the generation of S^0 and sulfide (S^{2-}) during the infection of *Verticillium* and *Fusarium* in tomato increases plant resistance (Williams et al., 2002b; Z. L. Zheng & Leustek, 2017). In this manner, maize plants could detect CgEP3 and induce defense responses against *C. graminicola* attack.

CgEP3 could affect selenium metabolism by suppressing *SELENBP1*. They observed that when the homologous gene in rice (*OsSBP*) is expressed constitutively in rice plants, it improves resistance against the fungus *M. oryzae* and the bacteria *X. oryzae* pv. *oryzae,* causing rice blast

and rice bacterial blight diseases, respectively (Sawada et al., 2004). Based on studies in *A. thaliana,* SBP1 plays a role in detecting the redox disequilibrium in the cells (Valassakis et al., 2018) and it could be implicated in general stress responses (Dervisi et al., 2020; Nikiforova et al., 2003). Thus, CgEP3 could counteract the maize plant defense response through the inhibition of SELENBP1.

Carbohydrate metabolism participates in the defense response of plants (Koch, 1996; Zhi-ming et al., 2021). CgEP3 could suppress two genes that are involved in the glycolytic pathway: enolase and triosephosphate isomerase (TPI). Enolase has been implicated in many plant-pathogen interactions (Y. Ding et al., 2019; Kaffamik et al., 2009; Mulema et al., 2011; Fan Zhang et al., 2019; Yixiang Zhang et al., 2013). Homolog of TPI in rice is expressed against several stresses: induced by wounding (at 6–24 hat), low temperature, and H_2O_2 stresses (6 hat), and suppressed in case of heat, drought, and NaCl (6–24 hat) (Sharma et al., 2012) CgEP3 could suppress both proteins, which are essential for carbon metabolism and many downstream processes involved in plant defense.

Interestingly, CgEP3 also affects lipid metabolism through the suppression of one gene that encodes the SMO1 enzyme, which is related to the phytosterol biosynthesis pathway. Sterols are structurally lipophilic molecules found in the majority of eukaryotic cell membranes (Gulati et al., 2010). Sterols and phospholipids are responsible for preserving membrane fluidity (Lagace & Ridgway, 2013). SMO1 could be differentially expressed during maize infection against *F. verticillioides* (upregulated) (Lambarey et al., 2020) and *C. heterostrophus* at 24 hpi (downregulated) (Y. Ding et al., 2019). CgEP3 might affect this pathway, which may be involved in maize defense against *C. graminicola.*

Similarly, CgEP3 could induce two genes involved in iron metabolism: *DMAS1* and *BOLA1.* Iron is an important element for a large number of organisms. Pathogens and hosts often compete for this nutrient. The role of iron during host-pathogen interaction is intricate. Iron can be used by plants to increase oxidative stress during the defense response, the liberation of phenolic compounds, etc. Certain components, such as iron transporters or iron storage proteins, help in the specific localization of iron and the defense response during infection (Aznar et al., 2015). *DMAS1* participates in iron phytosiderophore biosynthesis, which could be involved in plant interactions with pathogens. *DMAS1* is downregulated in maize (Meyer et al., 2017), and its expression in rice is predicted to be correlated with partial resistance against *M. oryzae* (Grand et al., 2012). Based on *A. thaliana* studies, the plastidial BOLA1 protein (Couturier et al., 2014) is a targeting factor involved in iron-sulfur (Fe-S) cluster trafficking (Przybyla-Toscano et al., 2021). It is downregulated against *B. cinerea* infection in *A. thaliana* (Whitley, 2019). Because we have seen that iron is important for plant defense, maize could respond to CgEP3 and induce the expression of iron-related genes. This could improve the immune response to *C. graminicola* infection.

Plants generate a great variety of low molecular weight compounds, including secondary metabolites (Erb & Kliebenstein, 2020; Taiz et al., 2015). Secondary metabolites often belong to a specific plant lineage and help plants interact with biotic and abiotic environments. The most abundant metabolites belong to the group of phenols, terpenes, and compounds that contain nitrogen (Erb & Kliebenstein, 2020; T. Hartmann, 2007) One of the most important pathways is the biosynthesis of phenylpropanoid compounds and it is related to plant defense. CgEP3 could suppress two enzymes related to this pathway, ATD and OMT, and indirectly suppress PKS1 based on studies of homologous maize genes in *A. thaliana.* PKS1 may be related to the CHS enzyme (Pan, 2012). This enzyme participates in the biosynthesis of naringenin chalcone, which

is the precursor of naringenin. This compound could act as an ATD enzyme in the formation of hesperetin, or downstream OMT, which converts apigenin into genkwanin, which is a precursor of salvigenin (Agostini et al., 2021; Portwood et al., 2019; Wen et al., 2014). The importance of the phenylpropanoid pathway in maize resistance against anthracnose fungus has been often reported (Balmer et al., 2013; Bhat et al., 2019a; de Jesus Miranda et al., 2017). Balmer et al. reported that high concentrations of flavonoids, such as naringenin chalcone and apigenin, played an important role in local defense response of maize leaves and root infected by *C. graminicola*. Additionally, apigenin, genkwanin, and chlorogenic acid decreased the radial growth of the fungus, depending on the dose of these compounds. Taking these results together, Balmer et al. concluded that maize has a wide variety of chemical compounds that help fight infection (Balmer et al., 2013; de Jesus Miranda et al., 2017). Balmer et al. reported that high concentrations of flavonoids, such as naringenin chalcone and apigenin, played an important role in local defense response of maize leaves and root infected by *C. graminicola*. Additionally, apigenin, genkwanin, and chlorogenic acid decreased the radial growth of the fungus, depending on the dose of these compounds. Taking these results together, Balmer et al. concluded that maize has a wide variety of chemical compounds that help fight infection (Balmer et al., 2013; de Jesus Miranda et al., 2017). In this way, CgEP3 could suppress the biosynthesis of these compounds, which are important for the maize defense response against *C. graminicola.* CgEP3 could counteract plant immunity at this point to improve disease development. On the other hand, CgEP3 could induce the expression of TPS6, which participates in the production of monocyclic sesquiterpenes β-bisabolene and β-macrocarpene. The last compound participates in the biosynthesis of zealexin (Huffaker et al., 2011; Köllner et al., 2008) which are non-volatile sesquiterpenoid phytoalexins that are effective in plant defense responses against different species of pathogenic fungi, such as *C. sublineolum*, *F. graminearum*, *C. heterostrophus*, *A. flavus*, and *Rhizopus microsporus*. It also has a moderate response in *C. graminicola* (Christensen et al., 2018; Huffaker et al., 2011). TPS6 is also elicited in response to *F. verticillioides* during the early stages of infection (Wang et al., 2016b) and *C. Zeina* (Meyer et al., 2017). Because TPS6 is important for plant defense against fungal pathogens, maize could respond to CgEP3 and induce its expression. This fact could enhance its role in the immune response of *C. graminicola.*

RNA metabolism is important in plant defense. There are a group of proteins that interact with RNA that have been designated as RNA-binding proteins (RBPs) (Burjoski & Reddy, 2021). Plant RBPs participate in different general processes, such as growth and development, genome organization, responses to various stresses, plant defense responses, mRNA processing, and post-transcriptional regulation (Burjoski & Reddy, 2021; Marondedze, 2020). Thus, CgEP3 could suppress four genes associated with RNA metabolism. The SR protein family is involved in the splicing process of pre-mRNA (Staiger & Brown, 2013; Witten & Ule, 2011). Little is known about the SR proteins that are related to biotic stress. One example is the splicing factor Ad-RSZ21 of *Arachis diogoi.* It is an SR protein that is associated with At-RSZ22 from *A. thaliana,* which plays a role in the plant defense response by inducing a hypersensitive response leading to cell death. It also participates in the up-regulation of different transcripts implicated in defense (Staiger & Brown, 2013; Trivedi et al., 2013) (K. R. R. Kumar & Kirti, 2012; Staiger & Brown, 2013). Another gene that could be suppressed by CgEP3 was *ZmPPR310.* PPR proteins in chloroplasts and mitochondria are essential at different stages of RNA metabolism, such as endonucleolytic processing, splicing, editing, and translation initiation (Barkan & Small, 2014; Manavski et al., 2018; Shikanai & Fujii, 2013). There are some examples of how PPRs may be involved in maize immune response: several PPR genes were differentially expressed under fungal *U. maydis* infection (Wei & Han, 2016), and *ZmPPR310* is downregulated when maize is infected with *C.*

Zeina (Christie et al., 2017). On the other hand, CgEP3 could affect the subunits of mitochondrial and cytoplasmic ribosomes by suppressing mitochondrial ribosomal protein S34 (mtS34) and RPL10A. The homologous gene of maize *mtS34* in *A. thaliana* encodes the mitoribosomal protein mS34. Another mitoribosomal protein exhibits alterations in developmental aspects when mutated (Robles & Quesada, 2017), but the action of *AtmtS34* in plant defense is unclear. The homologous gene of maize *RPL10A* in rice encodes OsL10a, which is involved in plant-pathogen interactions. It is associated with enhanced resistance to rice from bacterial blight by *X. oryzae* pv. *oryzae* (Jung et al., 2014). Homologs of this gene in *A. thaliana* is downregulated against *Agrobacterium tumefaciens* infection (Ditt et al., 2006). In summary, CgEP3 could affect RNA metabolism at different points, suppressing RBPs and ribosomal proteins, which could be important for plant defense. Thus, CgEP3 could improve host colonization during disease development.

Protein activity can be modulated through post-translational modifications essential for protein stability, such as ubiquitination, where the proteins are derived through the degradative pathway by the 26S proteasome. This system constitutes the most important pathway of protein degradation in cells (Zeng et al., 2006) and participates in numerous growth and development processes, as well as biotic and abiotic stress responses (F. Q. Xu & Xue, 2019; Zeng et al., 2006). CgEP3 suppresses two hypothetical U-box E3 ubiquitin ligases (*GRMZM2G384706* and *GRMZM2G449083*), which could be involved in ubiquitination and protein folding by molecular chaperones (Aravind & Koonin, 2000; Hatakeyama & Nakayama, 2003). Some U-box proteins are known to be involved in the defense response of plants against pathogens (González-Lamothe et al., 2006; Kirsch et al., 2001; Ren et al., 2014; B. Yang et al., 2006; Zeng et al., 2004), such as U-box genes that functioning against the fungal pathogen *M. oryzae* in rice. They could be implicated in the regulation of responses and signaling (Zeng et al., 2008). Two other genes can be suppressed by CgEP3, which belongs to the peptidyl prolyl cis/trans isomerase protein superfamily: *ZmFKBP17-3* and *ZmCYP33* (Qianqian Wang et al., 2017; Y. Yu et al., 2012). This family plays a role in various processes, such as protein folding (Y. Yu et al., 2012). Additionally, CYPs are molecular chaperones, which are involved in the proper folding of proteins, assembly, and stabilization (Hartl et al., 1994; Trivedi et al., 2012; Qianqian Wang et al., 2017). Some studies have shown that these genes are involved in plant immune responses (Y. Ding et al., 2019; Fabro et al., 2008). In contrast, CgEP3 induced the *DNAJC9* gene. Based on rice analysis, J-proteins/DnaJ proteins participate in basal cell processes, development, and stress. They are involved in protein folding and disaggregation, which are important during adverse conditions in cells (Sarkar et al., 2013). Proteins that are related to the folding of other proteins are important for defenses, such as the proteins of the ubiquitin-proteasome degradative pathway which are useful to maize during *C. graminicola* infection. This may help in replenishing cellular protein turnover as well as in the degradation of the effectors that have been secreted by the pathogen, reducing pathogenicity (Bhat et al., 2019). Vargas et al. (2012), also demonstrated that the stabilization of proteins was important during *C. graminicola* attack. The recycling of proteins could provide elements necessary for the biosynthesis of proteins related to plant diseases. In this way, CgEP3 suppressed these processes simultaneously to counteract maize defenses affecting U-box E3 ubiquitin ligases, ZmFKBP17-3 and ZmCYP33 proteins. At the same time, plants might also respond to CgEP3 and induce the expression of *DNACJ9* to improve the immune response to *C. graminicola* attack.

The structure and composition of the cell wall constantly change in response to the specific needs of the plant as well as diverse biological processes. The mechanism of maintenance of plant cell wall integrity (CWI) helps support the cytoskeleton during the growth of cell walls and

interactions with the environment and various pathogens (Gigli-Bisceglia et al., 2020). CgEP3 suppresses the maize CAZy GT47 family protein, which could be implicated in the cell wall remodeling process. Gigli-Bisceglia et al.found an example of this type of protein that is related to plant-pathogen interactions against *Blumeria graminis* f. sp. *hordei* infection of barley leaves. CAZy GT47 together with GT43 are predicted to be involved in xylan backbone biosynthesis. During infection, papillae deposition is produced. The heteroxylan of papillae could contribute to the resistance of the plant against penetration by the fungus (J. Chowdhury et al., 2017). However, it is necessary to select GT47 genes to describe their specific functions (Geshi et al., 2018). Nonetheless, CgEP3 could suppress this gene in maize, which could be important for plant cell wall integrity during *C. graminicola* infection. Fungus can suppress it to improve host colonization and disease development.

There are other genes that could be suppressed or induced by CgEP3 that have not been classified in any functional category. Among these genes, *CK1, SEY1, DEH2,* and *GRMZM2G035636* (phytepsin) were suppressed. CK1 is a protein kinase that participates in several processes, including stress responses (Kang & Wang, 2020). It is upregulated in maize infected with *C. heterostrophus* at 24 hpi (Y. Ding et al., 2019). Homologs of *SEY1* gene in *A. thaliana* is annotated as RDH3-like-2 which, together with RHD3, controls the tubule fusion in the endoplasmic reticulum (Stefano et al., 2014). Grégoire (2013) used heterologous microarray hybridization with a rice oligonucleotide array to measure gene expression in creeping bentgrass plants (*Agrostis stolonifera*) during infection with *Sclerotinia homeocarpa,* (producing the dollar spot disease) and reported that this gene was downregulated after pathogen infection. Dehydrins (DEHs) are a group of proteins that are normally expressed under certain stresses, such as drought and cold (Borovskii et al., 2002; Engelberth et al., 2019; Rorat, 2006). Korsman (2015) observed that maize *DEH2* takes part in a QTL that could control the growth of *C. zeina* during leaf infection, and Ding et al. (2019), reported that it is downregulated against *C. heterostrophus* at 24 hpi. Dehydrins can participate by controlling the hydric relationship between the host and pathogen (Jones et al., 2006). Similarly, phytepsins are related to the aspartic protease family that participates in biotic stress resistance (Yang & Feng, 2020) in different plant pathosystems (Sebastián et al., 2020; Y. Yang & Feng, 2020). Taken together, CgEP3 could affect multiple pathways by suppressing several genes that could be involved in plant defense.

In addition, CgEP3 induced the expression of *GRMZM2G085711, GRMZM2G045675, GRMZM2G090245,* and *GRMZM5G836353. GRMZM2G085711* encodes an apyrase protein. Based on *A. thaliana* studies, extracellular ATP is hydrolyzed by the action of apyrases and could play an important role in the defense response of the plant (J. Choi et al., 2014). When *AtAPY1* and *AtAPY2* (homolog of maize *GRMZM2G085711*) genes were suppressed, extracellular concentration of ATP started to increase. As a result, plants expressed numerous genes related to stress (J. Choi et al., 2014; Lin et al., 2014). Some studies support the function of apyrases during biotic stresses in plants (Cao, Tanaka, et al., 2014; Clark et al., 2014). The apyrase PsAPY1 was reported to impart immunity to pea plants by binding PsAPY1 to both a defense elicitor and a suppressor that were isolated from the pathogenic fungus *Mycosphaerella pinoides* (J. Choi et al., 2014; Kiba et al., 2006). Tobacco plants that constitutively expressed the *PsAPY1* gene showed resistance against the pathogens *Alternaria* sp. and *P. syringae* pv. *tabaci* (J. Choi et al., 2014; Shiraishi, 2013). On the other hand, *GRMZM2G045675* is annotated as dehydration related protein. Based on studies of its homologs in *A. thaliana,* this gene could be implicated in the activation of defense-related genes (Rama Devi et al., 2006). This gene responds to several viral infections in *A. thaliana*. Microarray data from 11 species of viruses that infect *A. thaliana*

were analyzed by Postnikova & Nemchinov (2012). *GRMZM2G090245* is annotated as a germin-like protein subfamily 3 member 3. Germin-like proteins can participate in important processes in plants, such as defense (Ilyas et al., 2016). This gene and its homologs in rice are differentially expressed in distinct hosts against fungal pathogens (Mulema et al., 2011; Shu, 2014; Z. Yuan et al., 2018). *GRMZM5G836353* is predicted to encode monooxygenase 1 (Shi et al., 2019) which could be implicated in plant immunity. It is upregulated in *C. heterostrophus* at 24 hpi (Y. Ding et al., 2019), and its homologous gene in *A. thaliana* is expressed in the presence of the necrotrophic fungus *A. brassicicola* (Mukherjee et al., 2009). Thus, it may be presumed that maize may respond to CgEP3, which in turn, can induce these genes to improve immune response of maize to *C. graminicola* infection, since the genes are already known to play vital roles in plant defense.

There are several genes with unknown functions on which little or no information was available either in the databases or in any existing literature. Among these genes *GRMZM2G145563*, *GRMZM2G164963*, *GRMZM2G092758*, *GRMZM2G517065*, *GRMZM2G153635* and *GRMZM2G052255* are suppressed by CgEP3, while *GRMZM2G029810*, *GRMZM5G813709* and *GRMZM2G056216* are induced by CgEP3. *GRMZM2G145563* appears to be upregulated in the maize resistant line RIL387 during *C. zeina* leaf infection (Meyer et al., 2017). Homologs of *GRMZM2G164963* in *A. thaliana* could be related to *VTE6* (phytyl phosphate kinase), which plays a role in tocopherol biosynthesis in chloroplasts (Plohmann, 2015). Tocopherols are fat-soluble antioxidant compounds that participate in the defense of plants against biotic and abiotic stresses ((Cela et al., 2018; Munné-Bosch, 2005; Zaid & Wani, 2019). In summary, CgEP3 could affect these groups of genes by either suppressing or inducing them. These unknown genes could be important for maize plant defense against *C. graminicola* and may represent new findings related to plant immunity.

Finally, we further investigated plant defense responses related to reactive oxygen species (ROS) burst. During the attack of plant pathogens, one of the first responses is the accumulation of ROS at the infection sites (Apostol et al., 1989; Nürnberger et al., 2004; Vargas et al., 2012). In *C. graminicola*, ROS generation is elevated and persistent in plant cells as the infection progresses. During the early stages of infection, maize cells produce oxidative vesicles that activate the expression of genes associated with the defense response (Vargas et al., 2012). Although we did not find genes directly related to ROS burst, CgEP3 could affect different processes that are indirectly linked. Maize could induce genes related to sulfur, carbon, and iron metabolism after the recognition of CgEP3. Sulfur compounds can play a role in the detoxification of high concentrations of ROS. For example, GSH is necessary for the alleviation of ROS stress (Capaldi et al., 2015; Noctor et al., 2012; Z. L. Zheng & Leustek, 2017). In the same way, sugars can increase oxidative burst, lignification of plant cell walls, and stimulate the biosynthesis of pathogenesis-related proteins or flavonoids during the early stages of infection (Morkunas & Ratajczak, 2014). Homologs of maize triosephosphate isomerase in *A. thaliana* (*AT3G554440.1*) could be important for redox-sensitivity during the early response after H_2O_2 treatment. After studying oxidative stress in the yeast model system, it was shown that the inhibition of glycolysis could be due to the metabolic flux directed towards the pentose phosphate pathway. The generated NADPH is used by the antioxidant system in cells (Ralser et al., 2007; Shenton & Grant, 2003; H. Wang et al., 2012). With respect to iron metabolism, this element participates in the production of harmful ROS; thus, the plant can use iron to improve the local stress produced by the accumulation of ROS during the defense response against the attack of pathogens (Aznar et al., 2015). Based on *A. thaliana* studies, BolA-like proteins may control redox reactions (Couturier et al., 2014; Mostafa et al., 2016). In turn, the activity of BolA-

like proteins is redox controlled by the interaction of monothiol glutaredoxins found in the same cell compartment (Couturier et al., 2014; L. Qin et al., 2015). CgEP3 induces a germin-like protein (GLP) which are a part of the "cupin" superfamily of proteins. These proteins can participate in important processes in the plant, such as growth, development, and defense. GERs can be transported from the rupture of oxalate (oxalic acid), the products of which are H_2O_2 and CO_2 (Ilyas et al., 2016). CgEP3 suppresses SELENBP1. Homologs of this gene in *A. thaliana* (*AtSBP1*) play a role in detecting redox disequilibrium in the cells (Valassakis et al., 2018). In summary, CgEP3 indirectly influences the ROS burst process. Plants can recognize CgEP3 and induce several mechanisms that could improve the defense response. Based on *A. thaliana* studies, CgEP3 suppressed redox disequilibrium through SELENBP1.

CONCLUSION

Transcriptome analysis showed 56 differentially expressed genes that are responded against CgEP3. These are referred to as effector-responsive genes. There were 35 upregulated and 21 downregulated DEGs in plants infected with the mutant strains at 24 hpi. This means that there are 35 and 21 DEGs that could be suppressed or induced, respectively, by the action of CgEP3 when maize is infected with the wild-type strain of *C. graminicola* at 24 hpi. We have presented a summary of the processes that could be affected by CgEP3 action **(Figure 60)**. CgEP3 might counteract plant defense responses through several pathways, while maize can recognize the CgEP3 effector and activate others to improve plant immunity.

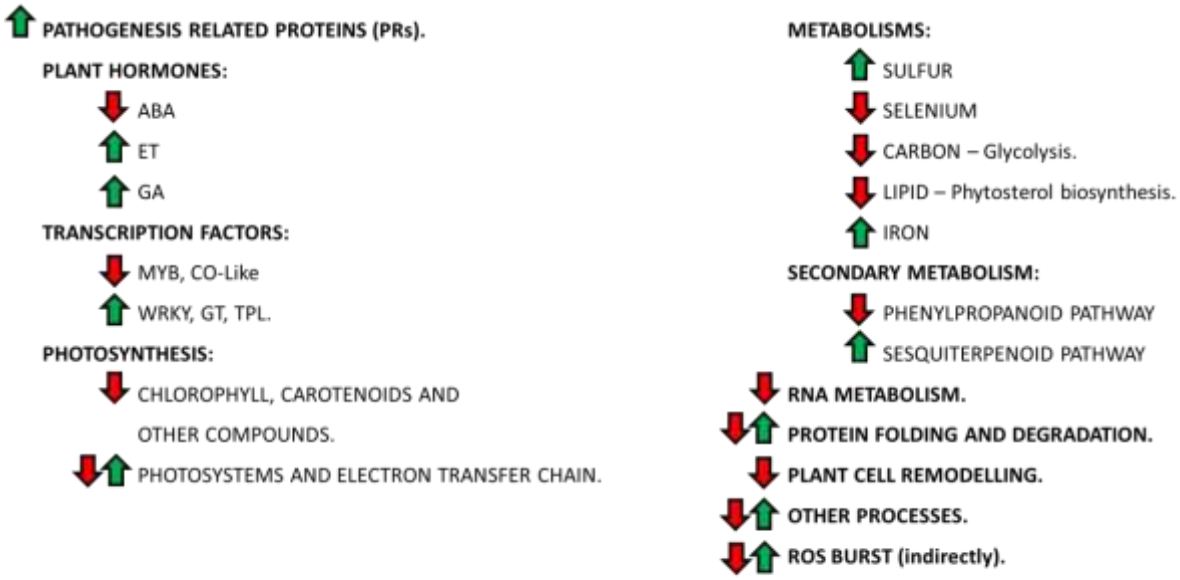


Figure 60. Summary of processes that could be affected by CgEP3. It may counteract plant defense response through several independent pathways while maize could recognize CgEP3 effector and activate others to improve plant immunity during the early stages of infection. Green and red arrows with black borders represent the pathways that are induced or suppressed by CgEP3, respectively.

In summary, two pathogenesis-related proteins were activated. Regarding plant hormones, the ABA pathway was suppressed, while the ET and GA pathways were induced. Several transcription factors were suppressed (MYB and CO-Like) or induced (WRKY, GT, and TPL). Photosynthesis-related genes such as chlorophyll, carotenoids, and other biosynthesis

compounds were suppressed, while components of the photosynthetic apparatus, such as photosystems and electron transfer chain, were suppressed or induced. With respect to metabolism, genes belonging to sulfur and iron metabolism were induced, whereas selenium, carbon (glycolysis), and lipid (phytosterol biosynthesis) were suppressed. Regarding secondary metabolism, certain genes related to the phenylpropanoid pathway were suppressed, and other sesquiterpenoid phytoalexin pathways were induced. Some genes involved in RNA metabolism are suppressed. In connection with protein folding and degradation, genes were suppressed or induced. With respect to plant cell wall remodeling, there was a gene that was suppressed. There are other important processes in which the genes are suppressed or induced. Although we did not find genes directly related to ROS burst, CgEP3 could affect different processes that were indirectly linked. Together, these genes represent several independent defense mechanisms that are suppressed or induced by CgEP3 at the early stages of infection by *C. graminicola.*

In the same way, many of these proteins have been predicted to localize to the nucleus and other cellular compartments. CgEP3 was released by *C. graminicola* and translocated into the cytoplasm of plant cells. It could be imported to the nucleus where it might alter different cellular processes related to plant defense. One example of *a C. graminicola* nuclear effector was CgEP1. It is translocated to the maize nucleus to affect genes that play a role in plant defense responses (Vargas et al., 2016). At present, the number of identified nuclear effectors of plant pathogenic fungi, as well as the signaling components of the plant immune response, are increasing. The great variety of nuclear effectors of fungi and other plant pathogens makes it possible to indicate the diversity of the regulatory networks of the plant defense response with which effectors can interact (Vargas et al., 2016).

Due to the effects of climate change and the increasing demand for maize worldwide, anthracnose of maize produced by *C. graminicola* results in a hazard to agriculture. Therefore, knowledge of host-pathogen interactions is vital for the production of new tools for the control of this disease (Balmer et al., 2013). Mechanisms that could be suppressed or induced by CgEP3 could provide new insights into *C. graminicola* control. Strategies based on constitutive expression and deletion of genes are important for defense signaling, which could be used in genetic engineering to improve the resistance of maize against the pathogen *C. graminicola* (de Jesus Miranda et al., 2017; Stuiver & Custers, 1928). Pharmacological treatments can be developed to inhibit the action of these effectors. They could be specific and safe to human and animal health, as well as successful for the control of anthracnose (Vargas et al., 2016), and importantly, in maize crops, CgEP3 is found only in the species *C. graminicola.* Future work will corroborate the expression patterns of some DEGs through quantitative real-time PCR (qRT-PCR) to validate the results obtained by RNA-Seq analysis.

MATERIALS AND METHODS:

Transcriptome analysis: mRNA libraries were obtained from maize leaf tissue samples 24 h after their inoculation with *C. graminicola* M1.001 wild-type strain, Δ*CgEP3* null mutant strain, and mock (Vargas et al., 2012). We performed three biological replicates of the experiment. The samples were sequenced using an Illumina HiSeq 2500 (Illumina Inc. San Diego, CA, USA). For RNA-Seq analysis, sequences were processed using the following procedure: read libraries were examined with FastQC, a quality control tool for high throughput sequence data analysis (Andrews, 2010)an. For mapping, the reads were aligned with a hybrid genome composed of

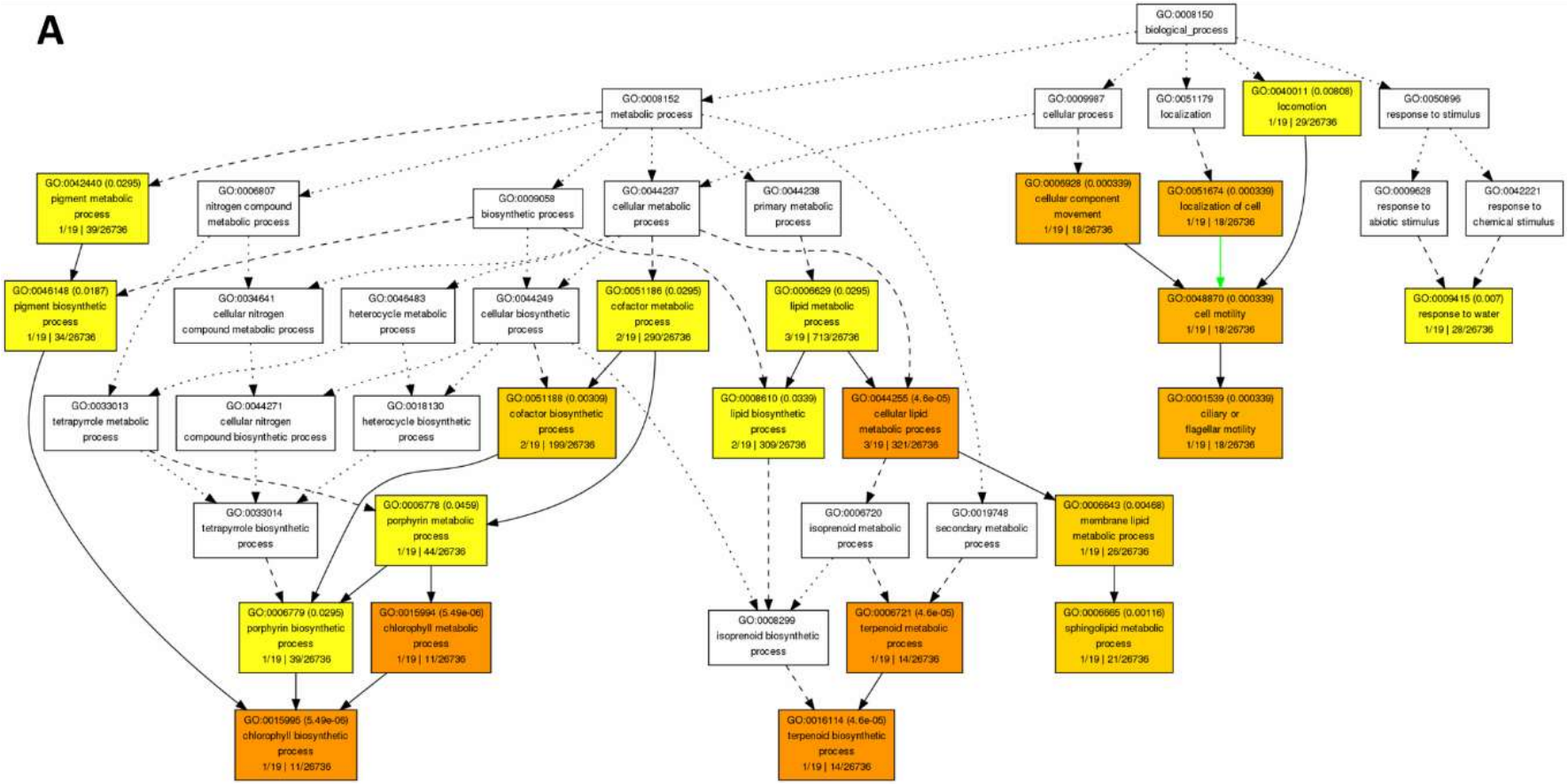
the *Zea mays* reference genome (B73_RefGen_v3) (Portwood et al., 2019) and *C. graminicola* (strain M1.001_V1) genome (R. J. O'Connell et al., 2012) using HiSat2 (v2.1.0) (D. Kim et al., 2019) For the treatment of BAM and SAM files, we used SamTools and SamTools Short (v.1.10) as a command line (Li et al., 2009). For each library, the number of reads mapped for each gene was counted using StringTie -eB (v.1.3.5) (Pertea et al., 2015). We calculated the abundance of reads and estimated the gene expression levels. Differential expression analysis between several pairwise comparisons and statistical analysis was performed using R Studio (RStudio Team, 2015) with DESeq2 R package (v.1.28.1) (Love et al., 2014). We used pre-filtering to keep ≥10 and $\alpha = 0.05$. Differentially expressed genes (DEGs) were designated as those where $\log_2$foldchange was ≥2 or ≤-2, and $p_{adjusted}$ was <0.05. Finally, to annotate the genes, we manually searched existing literature and public databases such as BLAST-Koala (KEGG), (Kanehisa et al., 2016), Gene Ontology (GO) (Carbon et al., 2019; Gene & Consortium, 2000, InterPro (Jones et al., 2014; Mitchell et al., 2019), PfamScan (Mistry et al., 2007); Phytozome 10 (Goodstein et al., 2012); Panther (Mi et al., 2013; P. D. Thomas et al., 2003); KOG (Bioinformatics et al., 2003) and MaizeGDB (Portwood et al., 2019).

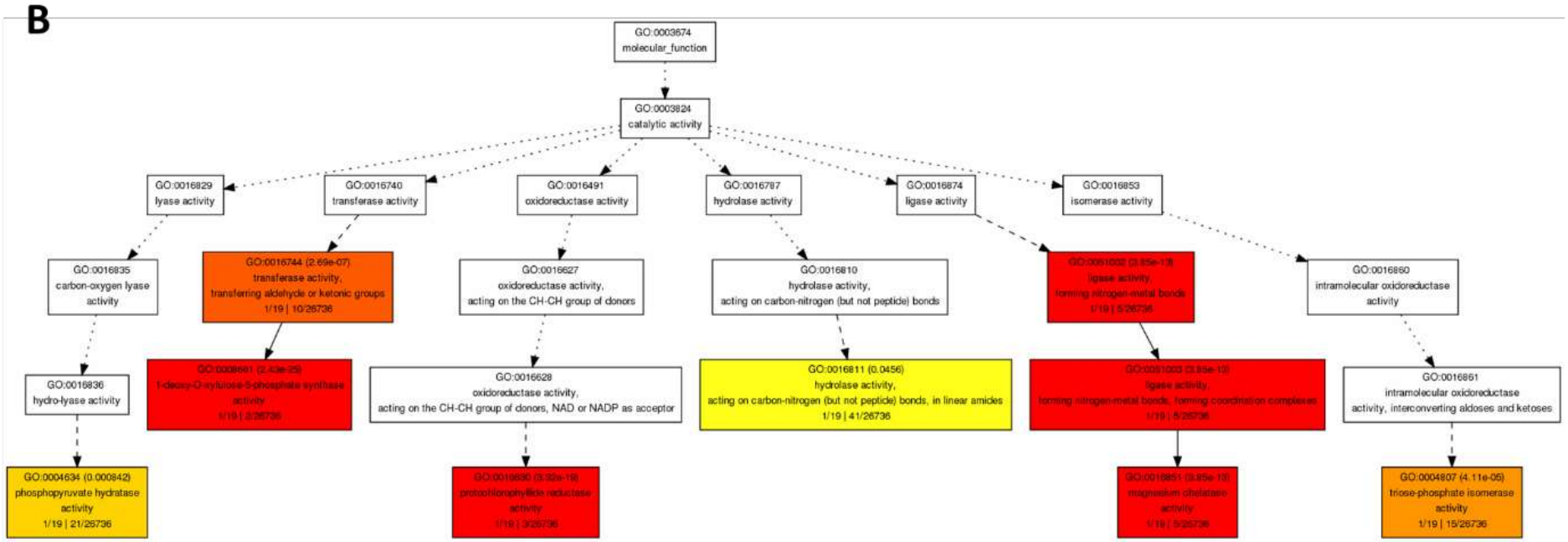
Gene Ontology Enrichment: The transcripts obtained in the RNA-Seq profile were subjected to gene ontology analysis using the agriGO singular enrichment analysis tool (Du et al., 2010b; T. Tian et al., 2017) and enriched GO terms were obtained by employing the Fisher test statistic (5% significance level) and the Yekutieli FDR multi-test adjustment method. The results included diverse biological, cellular, and molecular functions (Bhat et al., 2019b).

Subcellular localization of genes responsive to CgEP3 effector: We performed *in silico* analysis to predict subcellular localization using the sequence proteins encoded by 56 DEGs obtained after RNA-Seq analysis. Secreted proteins were predicted using SignalP 5.0 (José Juan A Armenteros et al., 2019) . Proteins that predicted signal peptides were considered to be secreted. We used different applications, such as WoLF PSORT (Horton et al., 2007), Yloc (Briesemeister et al., 2010), BaCelLo (Pierleoni et al., 2006), Plant-mPloc (Chou & Shen, 2010), and TargetP 2.0 (Armenteros et al., 2019b), for general subcellular localization prediction into plant cells; ChloroP 1.1 (Emanuelsson et al., 1999) for chloroplast transit peptide prediction; NLS Tradamus (Nguyen Ba et al., 2009) and cNLS Mapper (Kosugi et al., 2009) for nuclear localization signal prediction; and NoD-Nucleolus (Scott et al., 2011) for nucleolar localization sequence prediction.

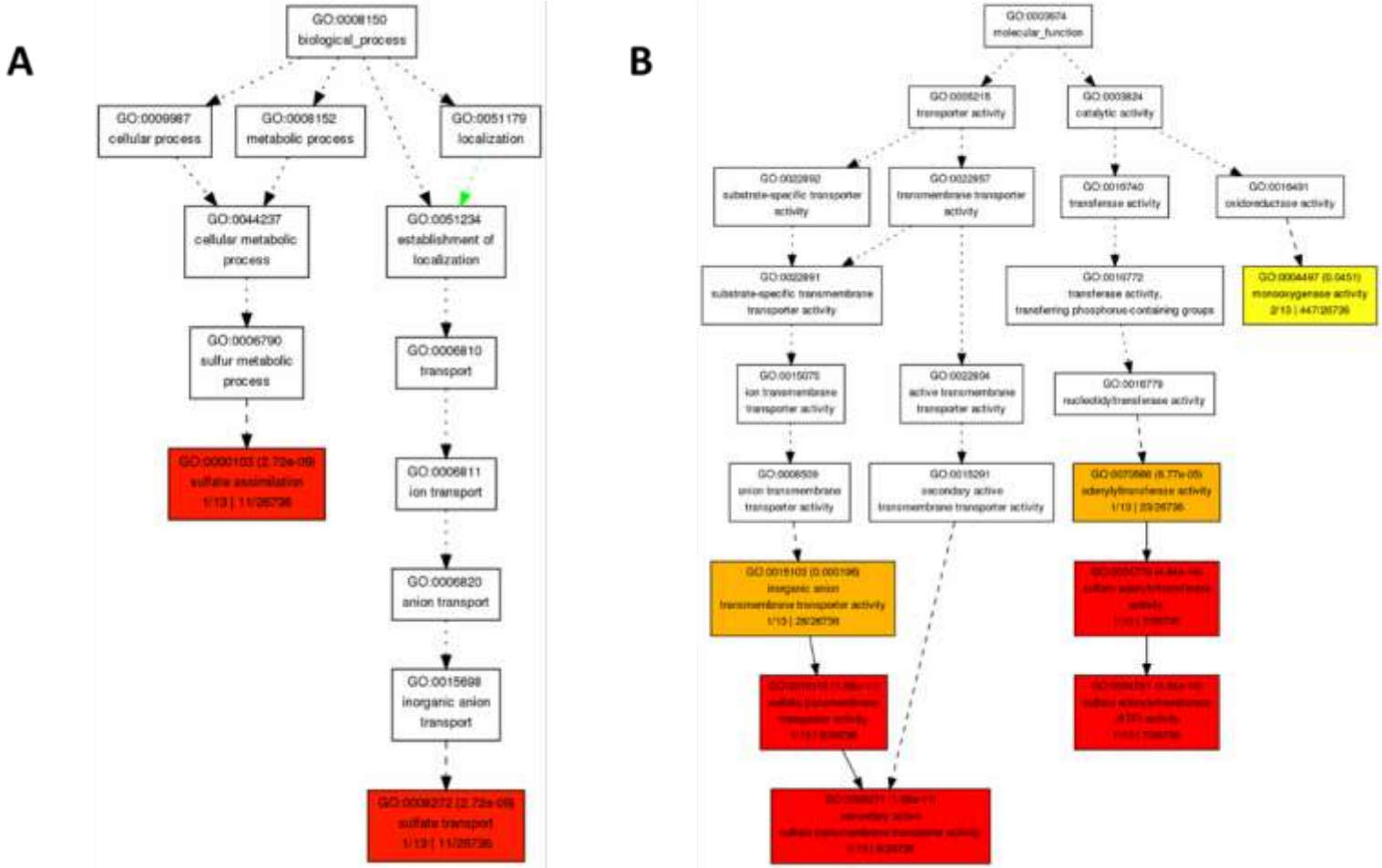
SUPLEMENTARY INFORMATION:

Supplementary Figure 4. Gene Ontology Enrichment Terms through agriGo (Du et al., 2010; Tian et al., 2017). 35 DEGs that are suppressed by CgEP3 are enriched in: **A)** Biological Process: “chlorophyll biosynthetic process”, “terpenoid biosynthetic process”, “cellular lipid metabolic process”, etc. **B)** Molecular Function: “1-deoxy-D-xylulose-5-phophate synthase”, “protochlorophyllide reductase activity”, “ligase activity”, “forming nitrogen-metal bonds”, etc.




B
GO:0003674
molecular_function
GO:0003824
catalytic activity
GO:0016829
lyase activity
GO:0016740
transferase activity
GO:0016491
oxidoreductase activity
GO:0016787
hydrolase activity
GO:0016874
ligase activity
GO:0016853
isomerase activity
GO:0016835
carbon-oxygen lyase activity
GO:0016744 (2.69e-07)
transferase activity, transferring aldehyde or ketonic groups
1/19 | 10/26736
GO:0016627
oxidoreductase activity, acting on the CH-CH group of donors
GO:0016810
hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
GO:0016860
intramolecular oxidoreductase activity
GO:0016836
hydro-lyase activity
GO:0008661 (2.43e-25)
1-deoxy-D-xylulose-5-phosphate synthase activity
1/19 | 2/26736
GO:0016628
oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor
GO:0016811 (0.0456)
hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides
1/19 | 41/26736
GO:0016861
intramolecular oxidoreductase activity, interconverting aldoses and ketoses
GO:0004634 (0.000842)
phosphopyruvate hydratase activity
1/19 | 21/26736
GO:0016630 (3.32e-19)
protochlorophyllide reductase activity
1/19 | 3/26736
GO:0004807 (4.11e-05)
triose-phosphate isomerase activity
1/19 | 15/26736

Supplementary Figure 5 . Gene Ontology Enrichment Terms through agriGo (Du et al., 2010; Tian et al., 2017). 21 DEGs that are induced by CgEP3 are enriched in: **A)** Biological Process: “sulfate assimilation” and “sulfate transport”. **B)** Molecular Function: “secondary active sulfate transmembrane transporter activity”, “sulfate adenylyltransferase (ATP) activity”, “monooxygenase activity”, etc.



SUPLEMENTARY INFORMATION:

Supplementary Table 7. CgEP3 effector responsive genes. Differentially expressed genes (DEGs) in RNA-seq experiments when maize leaves are inoculated with Δ*CgEP3* compared to WT treated leaves. Maize gene ID correspond to *Zea mays* reference genome (B73_RefGen_V3). Log2foldchange represents genes that are up-regulated or down-regulated, positive, and negative values, respectively. DEGs are considered when log2foldchange is ≥ 2 or ≤ -2, and $P_{adjusted}$ (Padj) is < 0.05. There are 56 DEGs which 35 are up-regulated (DEGs that could be suppressed in presence of CgEP3 effector in wild type inoculated leaves) and 21 DEGs that are down-regulated (genes that could be induced in presence of CgEP3 effector in wild type inoculated leaves). We have been searched into literature and database to find the best annotation. DEGs have been assigned to functional categories. Based in Phytozome 10 data of homologous genes that appeared in MaizeGDB database (Goodstein et al., 2012; Portwood et al., 2019), We have found homologous in another plant models as *A. thaliana* and rice. NA means non-available. An extension of this table has found in Supplementary Table 9.

MAIZE GENE ID	LOG2 FOLD CHANGE	PADJ	MAIZE ANNOTATION	REFERENCE	BEST HIT *A. THALIANA*	ANNOTATION	REFERENCE	BEST HIT RICE	ANNOTATION	REFERENCE
DEFENSE RESPONSE:										
GRMZM2G456997	-2,28	3,9E-04	Pathogenesis-related protein PRB1-3-like	Bath et al., 2019	AT4G33720.1	PR1, pathogenesis-related protein	Husaini et al., 2018	LOC_Os01g28450.1	OsPR1b, pathogenesis-related protein	Zhang et al., 2016
GRMZM2G125032	-2,25	4,9E-02	β-1,3-glucanase	Xie et al., 2014	AT4G16260.1	β-1,3-endoglucanase	Hamamouch et al., 2012	LOC_Os01g71670.1	glycosyl hydrolases family 17, putative, expressed	Bagnaresi et al., 2012
PLANT HORMONES:										
GRMZM2G150363	2,41	8,2E-03	NCED8, 9-cis carotenoid cleavage dioxygenase 8	Dutta et al., 2019	AT4G19170.1	9-cis-epoxicarotenoid dioxygenase	Pastori et al., 2003	LOC_Os02g47510.1	NCED, 9-cis-epoxycarotenoiddioxygenase	Wang et al., 2015
GRMZM2G370991	-10,06	2,9E-09	EIN2-like, Ethylene-Insensitive 2-Like protein	Mei et al., 2019	AT5G03280.1	EIN2, Ethylene-Insensitive 2 protein	Y. Zhang et al., 2020	LOC_Os07g06130.1	OsEIN2.1, Ethylene-Insensitive 2.1 protein	Salvador-Guirao et al., 2018
GRMZM2G147774	-3,83	9,0E-03	Cytochrome P450, E-class, group I	Jones et al., 2014; Mitchell et al., 2019	AT3G14690.1	Cytochrome P450 CYP72A15	He et al., 2019	LOC_Os01g43851.1	cytochrome P450 72A1 putative expressed	Goodstein et al., 2012
TRANSCRIPTION FACTORS:										
GRMZM2G049378	6,29	2,6E-03	ZmMYBR115	Gray et al., 2009	AT1G75250.1	PPR2-myb related protein	Federspiel et al., 2010	LOC_Os01g47370.1	MYB family transcription factor putative expressed	Goodstein et al., 2012

GRMZM2G107886	2,71	4,9E-04	ZmCOL16 (CONSTANS-LIKE 16)	Song et al., 2018	AT1G25440.1	BBX, B-Box domain protein 15	Barah et al., 2015	LOC_Os06g15330.1	zinc finger protein CONSTANS-LIKE 16	Lim et al., 2010
GRMZM2G169580	-2,83	2,2E-04	Triple-helix transcription factor family protein	Li et al., 2010	AT1G76890.2	AtGT2, trihelix transcription factor	Kaplan-Levy et al., 2012	LOC_Os02g43300.1	OsMLS15, trihelix transcription factor	J. Li et al., 2019
GRMZM2G381378	-2,71	6,7E-03	ZmWRKY83	Wei et al., 2012	AT3G56400.1	AtWRKY46	Hu et al., 2012	LOC_Os07g48260.1	OsWRKY47	Wei et al., 2013
GRMZM2G115701	-2,23	1,3E-04	ZmTPL6, TUBBY-like protein 6	Yulong et al., 2016	AT2G18280.1	AtTLP2, TUBBY-like protein 2	Takeda et al., 2019	LOC_Os02g47640.1	OsTPL8, TUBBY-like protein 2	Kou et al., 2009
PHOTOSYNTHESIS:										
GRMZM2G084958	2,61	4,1E-02	ZmPORA, protochlorophyllide reductase A	Kretschmer et al., 2017; Zhan et al., 2019	AT5G54190.1	PORA, protochlorophyllide reductase A	Yoo et al., 2020	LOC_Os04g58200.1	OsPORA, protochlorophyllide reductase A	Sakuraba et al., 2013
GRMZM2G351977	2,57	2,0E-03	ZmLHCBa, light-harvesting, chlorophyll a/b protein a	Allen et al., 2011; Dubreuil et al., 2018	AT2G34420.1	(LHB1B2, LHCB1.5) photosystem II light harvesting complex gene B1B2	Goodstein et al., 2012	LOC_Os01g41710.1	Chlorophyll a/b binding protein 2, chloroplast precursor, putative, expressed	Vergne et al., 2007
GRMZM2G137151	2,23	7,6E-03	DXS1, 1-deoxy-D-xylulose-5-phosphate synthase 1 protein	Zhang et al., 2019a	AT4G15560.1	AtDXS1, 1-deoxy-D-xylulose-5-phosphate synthase 1	Luna-Valdez et al., 2020	LOC_Os05g33840.1	Transketolase, putative, expressed	Kumari et al., 2017
GRMZM2G070847	2,22	2,9E-02	DNA translocase	Tang et al., 2018	AT1G21500.1	PI3KIA, phosphatidylinositol 3-kinase class IA	Han, 2017	LOC_Os09g34140.1	expressed protein	Goodstein et al., 2012
GRMZM2G419806	2,14	9,0E-05	CHLD, magnesium protoporphyrin IX chelatase protein	Zhang et al., 2019	AT5G45930.1	(CHL I2, CHLI-2, CHLI2) magnesium chelatase I2	Goodstein et al., 2012	LOC_Os03g36540.1	magnesium-chelatase subunit chlI chloroplast precursor putative expressed	Goodstein et al., 2012

GRMZM2G036880	2,13	3,2E-02	LHCA1; light-harvesting complex I chlorophyll a/b binding protein 1	Kanehisa et al., 2016	AT3G54890.1	LHCA1, photosystem I light harvesting complex gene 1	Jia et al., 2020	LOC_Os06g21590.1	Chlorophyll A-B binding	Buffon et al., 2016
GRMZM2G450705	-3,15	2,7E-02	NAD(P)H-quinone oxidoreductase subunit 2 (NdhB)	Kanehisa et al., 2016	ATCG00890.1	NdhB, NAD(P)H dehydrogenase-like complex, B	Laughlin et al., 2019	LOC_Os08g15248.1	NADH dehydrogenase B/A NdhB	F. Zhang et al., 2016
METABOLISM:										
GRMZM2G058930	20,87	1,5E-05	SELENBP1; methanethiol oxidase [EC:1.8.3.4]	Kanehisa et al., 2016	AT4G14030.1	AtSPB1	González-Gordo et al., 2020	LOC_Os01g68770.1	OsSBP	Sawada et al., 2004
GRMZM2G446253	8,03	2,6E-03	ENO, eno; enolase [EC:4.2.1.11]	Kanehisa et al., 2016	AT2G36530.1	Enolase	Kaffamik et al., 2009	LOC_Os06g04510.1	Enolase	Fan Zhang et al., 2019
GRMZM2G439389	7,71	9,0E-03	Triosephosphate isomerase (TPI)	N. Zhang et al., 2015	AT3G55440.1	Triose-phosphate isomerase	Kaffarnik et al., 2009	LOC_Os01g62420.1	OscTPI, triose phosphate isomerase	Sharma et al., 2012
GRMZM2G062531	4,34	2,0E-02	SMO1; plant 4,4-dimethylsterol C-4alpha-methyl-monooxygenase [EC:1.14.18.10]	Kanehisa et al., 2016	AT4G12110.1	SMO1, sterol-4alpha-methyl oxidase 1-1	Lange et al., 2020	LOC_Os10g39810.1	C-4 methylsterol oxidase	Fu et al., 2019
GRMZM2G088348	-8,33	5,2E-04	ATPS, ATP Sulfurylase	Portwood et al., 2018	AT5G43780.1	ATPS4, ATP Sulfurylase 4	Akbudak & Filiz, 2019	LOC_Os03g53230.1	OsATPSa, ATP Sulfurylase	Akbudak and Filiz, 2019
GRMZM2G132875	-3,36	1,1E-04	DMAS1, 3''-deamino-3''-oxonicotianamine reductase	Kanehisa et al., 2016	AT1G59960.1	NAD(P)-linked oxidoreductase superfamily protein	Goodstein et al., 2012	LOC_Os04g37490.1	Oxidoreductase	Grand et al., 2012
GRMZM2G172239	-2,23	6,8E-05	BOLA1; BolA-like protein 1	Kanehisa et al., 2016	AT1G55805.1	BOLA1	Przybyla-Toscano et al., 2021	LOC_Os03g11990.1	bolA putative expressed	Goodstein et al., 2012
GRMZM2G342907	-2,14	4,5E-02	ZmSULTR1.2b, putative sulfate transport protein	Chorianopoulou et al., 2020	AT1G22150.1	SULTR1;3, sulfate transporter	Zheng & Leustek, 2017	LOC_Os03g09970.1	OsSultr1;1, sulfate transport protein	Srivastava et al., 2016
SECONDARY METABOLISM:										

GRMZM2G424857	7,14	2,0E-03	ATD, acetamidase/formamidase family protein	Wen et al., 2014	AT4G37560.1	IAMH2, putative Acetamidase/formamidase	Gao et al., 2020	LOC_Os01g55950.1	Acetamidase (putative)	(Du et al., 2020
GRMZM2G085924	3,48	6,7E-03	OMT, O-methyltransferase	Liu et al., 2019	AT4G35150.1	O-methyltransferase family 2	Lapcik et al., 2006	LOC_Os11g20090.1	O-Methyltrasferase ZRP4, putative, expressed	Chakrabarty et al., 2009
GRMZM2G066291	2,77	3,2E-02	PKS1, Phytochrome Kinase Substrate 1	Song et al., 2017	AT1G18810.1	PKS3, Phytochrome Kinase Substrate 3	Legris et al., 2021	LOC_Os10g26670.1	Expressed protein	Goodstein et al., 2012
GRMZM2G127087	-2,79	2,4E-02	TPS6, Terpene synthase 6	Block et al., 2019	AT1G70080.1	AtTPS6, Terpene synthase	Wang et al., 2016	LOC_Os01g23530.1	Terpene synthase	Nanda et al., 2020
RNA METABOLISM:										
GRMZM2G137596	10,56	8,8E-10	Mitochondrial 28S ribosomal protein S34	Jones et al., 2014; Mitchell et al., 2019	AT5G52370.1	AtmS34	Tomal et al., 2019	LOC_Os02g27960.1	Expressed protein	Goodstein et al., 2012
GRMZM5G865151	7,68	3,0E-02	SR protein related family member, putative, expressed	Goodstein et al., 2012	AT1G61170.1	Unknown protein	Yan et al., 2016	LOC_Os01g57150.1	SR protein related family member	Wenhui et al., 2019
GRMZM2G074769	7,51	3,2E-02	RP-L10Ae, RPL10A; large subunit ribosomal protein L10Ae	Kanehisa et al., 2016	AT1G08360.1	RPL10aA, Ribosomal protein L10aA	Firmino et al., 2020	LOC_Os08g44450.1	OsL10a, putative 60S ribosomal protein L10	Im et al., 2011
GRMZM2G421231	2,64	6,7E-03	ZmPPR310, pentatricopeptide repeat protein 310	Wei and Han, 2016	AT1G09900.1	Pentatricopeptide repeat-containing protein	Paudel et al., 2016	LOC_Os01g57410.1	Pentatricopeptide repeat domain containing protein	P. Zhang et al., 2019
PROTEIN FOLDING AND DEGRADATION:										
GRMZM2G384706	7,53	3,4E-02	U-box domain containing protein, expressed	Goodstein et al., 2012	NA	NA	NA	LOC_Os03g13010.1	Putative U-box E3 ubiquitin ligase	Ren et al., 2014
GRMZM2G449083	4,76	8,2E-03	LysH-CTLH-CRA-RING uncharacterized protein	(Jaureguibeitia, 2015	AT4G37880.1	LisH/CRA/RING-U-box domains-containing protein	Goodstein et al., 2012	LOC_Os06g38940.1	RMD5 homolog A, expressed	Xiang et al., 2020

GRMZM2G132811	2,52	2,2E-04	ZmCYP33, cyclophilin protein 33	Wang et al., 2017	AT1G74070.1	AtCYP26-2, cyclophilin protein 26-2	Romano et al., 2004	LOC_Os01g02080.1	OsCYP-1, cyclophilin protein 1	Trivedi et al., 2013
GRMZM2G057374	2,00	9,5E-03	ZmFKBP17-3, FK506-binding protein 17-3	Yu et al., 2012	AT1G18170.1	AtFKBP17-2, peptidyl-prolyl cis/trans isomerase 17-2 protein	Ifuku et al., 2010	LOC_Os03g50080.1	OsFKBP17-2, peptidyl-prolyl *cis/trans* isomerase 17-2 protein	Gollan & Bhave, 2010
GRMZM2G090689	-7,48	1,7E-02	DnaJ homolog subfamily C member 9 (DNAJC9)	Kanehisa et al. 2016	AT3G12170.1	Chaperone DnaJ-domain superfamily protein	Goodstein et al., 2012	LOC_Os02g10220.1	OsDjC17	Sarkar et al., 2013
PLANT CELL REMODELING										
GRMZM2G000976	7,48	3,5E-02	CAZy glycosyltransferase 47 family (GT47)	PlantCAZyme Database, Ekstrom et al., 2014	AT4G38040.1	CAZy glycosyltransferase 47 family (GT47)	Geshi et al., 2018	LOC_Os02g09430.1	CAZy glycosyltransferase family 47 (GT47)	Ekstrom et al., 2014
OTHER GENES:										
GRMZM2G095634	8,78	4,1E-05	CK1_Casein Kinase_1a.2 - CK1 includes the casein kinase 1 kinases, expressed.	Goodstein et al., 2012	AT2G25760.2	AtAEL1/AtMLK3	Chen et al., 2018; Kang and Wang, 2020; Wilson et al., 2021	LOC_Os01g54100.1	MLK3 (CK1, Casein Kinase 1 Family protein)	Kang et al., 2020
GRMZM2G407913	6,48	1,1E-04	protein SEY1 [EC:3.6.5]	Kanehisa et al., 2016	AT5G45160.1	RHD3-like 2 (Root hair defective 3-like 2)	Stefano et al., 2014	LOC_Os12g41170.1	Root hair defective 3 GTP-binding (RHD3) family protein	Grégoire, 2013
GRMZM2G147014	2,47	3,0E-02	DEH2, dehydrin protein	Engelberth et al., 2019	AT1G20440.1	OsjDHN2, *Oryza sativa* ssp. *japonica*, dehydrin protein 2	Verma et al., 2017	LOC_Os02g44870.1	COR47, dehydrin	Jones et al., 2006

GRMZM2G035636	2,37	8,8E-10	E3.4.23.40; phytepsin [EC:3.4.23.40] - Aspartic peptidases	Kanehisa et al., 2016	AT1G11910.1	AtAPA1, aspartic proteinase A1	Mazorra-Manzano et al., 2010	LOC_Os05g49200.1	Aspartic proteinase oryzasin-1	Divya et al., 2016
GRMZM2G085711	-10,98	1,9E-12	APY, Apyrase protein	Kanehisa et al., 2016	AT5G18280.1	AtAPY2, Apyrase protein 2	Choi et al., 2014	LOC_Os12g02980.1	Nucleoside-triphosphate putative	Goodstein et al., 2012
GRMZM2G045675	-8,10	1,6E-03	dehydration response related protein, putative, expressed	Goodstein et al., 2012	AT4G00750.1	Dehydration-responsive gene	Rama Devi et al., 2006	LOC_Os04g48230.1	Dehydration response related protein, putative, expressed	Goodstein et al., 2012
GRMZM2G090245	-3,33	2,7E-03	Germin-like protein subfamily 3-member 3	Mi et al., 2013; Thomas et al., 2003	AT1G72610.1	AtGLP1 germin-like protein 1	Goodstein et al., 2012	LOC_Os08g35750.1	OsGLP8-13, germin-like protein	Ilyas et al., 2016
GRMZM5G836353	-2,80	1,3E-06	Monooxygenase 1	Shi et al., 2019	AT4G15760.1	Monooxygenase/ aromatic-ring hydroxylase I (MOI)	Mukherjee et al., 2009	LOC_Os02g27200.1	Expressed protein	Goodstein et al., 2012
UNKNOWN:										
GRMZM2G145563	9,81	1,1E-08	Unknown	Meyer et al., 2017	NA	NA	NA	NA	NA	NA
GRMZM2G092758	7,41	4,4E-02	NA	NA	NA	NA	NA	NA	NA	NA
GRMZM2G517065	7,21	7,1E-03	NA	NA	NA	NA	NA	NA	NA	NA
GRMZM2G153635	3,03	1,7E-02	NA	NA	NA	NA	NA	NA	NA	NA
GRMZM2G164963	2,28	3,2E-02	Uncharacterized protein family Ycf49	Jones et al., 2014; Mitchell et al., 2019	AT4G22830.2	YCF49-like protein	Berardini et al., 2015	LOC_Os04g31010.1	Expressed protein	Huang et al., 2013
GRMZM2G052255	2,15	4,8E-12	NA	NA	NA	NA	NA	NA	NA	NA
GRMZM2G029810	-8,39	1,7E-04	NA	NA	NA	NA	NA	NA	NA	NA
GRMZM5G813709	-8,28	3,6E-04	NA	NA	NA	NA	NA	NA	NA	NA
GRMZM2G056216	-7,91	2,0E-03	NA	NA	NA	NA	NA	NA	NA	NA

Supplementary Table 8. ***In silico*** **subcellular localization prediction of CgEP3 effector responsive genes.** We have used different applications: SignalP 5.0 for signal peptide prediction (Armenteros et al., 2019); WoLF PSORT (Horton et al., 2007), YLoc (Briesemeister et al., 2010), BaCelLo (Pierleoni et al., 2006), Plant-mPloc (Chou & Shen, 2010), TargetP 2.0 (Armenteros et al., 2019b) for general subcellular localization prediction into plant cell; ChloroP 1.1 (Emanuelsson et al., 1999) for chloroplast transit peptides prediction; NLS Tradamus (Nguyen Ba et al., 2009) and cNLS Mapper (Kosugi et al., 2009) for nuclear localization signal prediction; and NoD-Nucleolus (Scott et al., 2011), for nucleolar localization sequences prediction. Legend: Y and N refer to "yes" or "no", namely, presence or absence, respectively. CW, Cell Wall; CHL, Chloroplast; CHLTP, chloroplast transfer peptide; CMB; Cell Membrane; CYT, Cytoplasm; ER, endoplasmic reticulum; EXT, Extracellular; GAP, Golgi apparatus; MIT, Mitochondrion; NA, non-available; NUC, Nucleus; OTH, other; PL, Plastid; SECP, Secretory Pathway; SP, Signal Peptide; TLTP, Thylakoid luminal transfer peptide; and V, Vacuole.

MAIZE GENE ID	MAIZE ANNOTATION	REFERENCE	SignalP 5.0	WoLF PSORT	YLoc	BaCelLo	Plant-mPLoc	TargetP 2.0	ChloroP 1.1	NLS Tradamus	cNLS Mapper	Nucleolus
DEFENSE RESPONSE:												
GRMZM2G456997	Pathogenesis-related protein PRB1-3-like	Bath et al., 2019	Y	CHL	SECP	SECP	VAC	SP	N	N	N	N
GRMZM2G125032	β-1,3-glucanase	Xie et al., 2014	Y	CHL	SECP	SECP	VAC	SP	N	N	N	N
PLANT HORMONES:												
GRMZM2G150363	NCED8, 9-cis carotenoid cleavage dioxygenase 8	Dutta et al., 2019b	N	MIT	CHL	CHL	CHL	OTH	N	Y	N	Y
GRMZM2G370991	EIN2-like, Ethylene-Insensitive 2-Like protein	Mei et al., 2019	N	PL	SECP	NUC	CHL, NUC	SP	N	N	N	N
GRMZM2G147774	Cytochrome P450, E-class, group I	Jones et al., 2014; Mitchell et al., 2019	N	CHL	CHL	SECP	ER, VAC	OTH	N	N	N	N
TRANSCRIPTION FACTORS:												
GRMZM2G049378	ZmMYBR115	Gray et al., 2009	N	NUC	NUC	NUC	NUC	OTH	N	N	N	N
GRMZM2G107886	ZmCOL16 (CONSTANS-LIKE 16)	Song et al., 2018	N	NUC	NUC	NUC	NUC	OTH	N	Y	N	Y
GRMZM2G169580	Triple-helix transcription factor family protein	Li et al., 2010	N	NUC	NUC	NUC	NUC	OTH	Y	N	N	Y
GRMZM2G381378	ZmWRKY83	Wei et al., 2012	N	NUC	NUC	NUC	NUC	OTH	N	Y	N	N
GRMZM2G115701	ZmTPL6, TUBBY-like protein 6	Yulong et al., 2016	N	NUC, CYT	NUC	NUC	NUC	OTH	N	N	N	N
PHOTOSYNTHESIS:												
GRMZM2G084958	ZmPORA, protochlorophyllide reductase A	Kretschmer et al., 2017; Zhang et al., 2019	N	CHL	CHL	CHL	CHL	CHLTP	Y	N	N	N
GRMZM2G351977	ZmLHCBa, light-harvesting, chlorophyll a/b protein a	Allen et al., 2011; Dubreuil et al., 2018	N	CHL	CHL	CHL	CHL	CHLTP	N	N	N	N
GRMZM2G137151	DXS1, 1-deoxy-D-xylulose-5-phosphate synthase 1 protein	Zhang et al., 2019c	N	CHL	CHL	CHL	CHL	CHLTP	Y	N	N	Y
GRMZM2G070847	DNA translocase	Tang et al., 2018	N	CHL	CHL	NUC	CHL, NUC	TLTP	Y	Y	N	N
GRMZM2G419806	CHLD, magnesium protoporphyrin IX chelatase protein	Zhang et al., 2019b	N	CHL	CHL	CHL	CHL	CHLTP	Y	N	N	N
GRMZM2G036880	LHCA1; light-harvesting complex I chlorophyll a/b binding protein 1	Kanehisa et al., 2016	N	CHL	CHL	CHL	CHL	CHLTP	Y	N	N	N
GRMZM2G450705	NAD(P)H-quinone oxidoreductase subunit 2 (NdhB)	Kanehisa et al., 2016	N	PL	MIT	CHL	CHL, PL	OTH	N	N	N	N

METABOLISM:												
GRMZM2G058930	SELENBP1; methanethiol oxidase [EC:1.8.3.4]	Kanehisa et al., 2016	N	CYT	CHL	SECP	CHL	OTH	N	N	Y	Y
GRMZM2G446253	ENO, eno; enolase [EC:4.2.1.11]	Kanehisa et al., 2016	N	CYT	CYT	CYT	CYT, N.	OTH	N	N	Y	Y
GRMZM2G439389	Triosephosphate isomerase (TPI)	Zhang et al., 2015a	N	NUC	MIT	NUC	CHL	OTH	N	N	Y	Y
GRMZM2G062531	SMO1; plant 4,4-dimethylsterol C-4alpha-methyl-moNoxygenase [EC:1.14.18.10]	Kanehisa et al., 2016	N	CHL	SECP	NUC	ER, VAC	OTH	N	N	N	Y
GRMZM2G088348	ATPS, ATP Sulfurylase	Portwood et al., 2018	N	CHL	CYT	NUC	NUC	OTH	N	N	N	N
GRMZM2G132875	DMAS1, 3''-deamiN-3''-oxonicotianamine reductase	Kanehisa et al., 2016	N	CYT	CYT	NUC	CHL	OTH	N	N	N	N
GRMZM2G172239	BOLA1; BolA-like protein 1	Kanehisa et al., 2016	N	MIT	MIT	CHL	CHL	MITTP	N	N	N	N
GRMZM2G342907	ZmSULTR1.2b, putative sulfate transport protein	Chorianopoulou et al., 2020	N	PL	CHL	CYT	CMB	OTH	N	N	N	N
SECONDARY METABOLISM:												
GRMZM2G424857	ATD, acetamidase/formamidase family protein	Wen et al., 2013	N	CYT	CYT	NUC	CHL	OTH	N	N	N	Y
GRMZM2G085924	OMT, O-methyltransferase	Liu et al., 2019	N	CYT	CYT	CYT	CHL	OTH	N	N	Y	N
GRMZM2G066291	PKS1, Phytochrome Kinase Substrate 1	Song et al., 2017	N	NUC	NUC	NUC	CMB	OTH	N	N	N	N
GRMZM2G127087	TPS6, Terpene synthase 6	Block et al., 2019	N	CYT, MIT	CHL	CHL	CHL	OTH	N	N	N	N
RNA METABOLISM:												
GRMZM2G137596	MIT 28S ribosomal protein S34	Jones et al., 2014; Mitchell et al., 2019	N	CHL	MIT	CHL	CHL	MITTP	N	Y	N	Y
GRMZM5G865151	SR protein related family member, putative, expressed	Goodstein et al., 2012	N	NUC	NUC	NUC	NUC	OTH	N	Y	Y	N
GRMZM2G074769	RP-L10Ae, RPL10A; large subunit ribosomal protein L10Ae	Kanehisa et al., 2016	N	CHL	CYT	NUC	CHL	OTH	N	Y	N	Y
GRMZM2G421231	ZmPPR310, pentatricopeptide repeat protein 310	Wei and Han, 2016	N	CHL	CHL	CHL	CHL	OTH	Y	Y	N	N
PROTEIN FOLDING AND DEGRADATION:												
GRMZM2G384706	U-box domain containing protein, expressed	Goodstein et al., 2012	N	CYT	CYT	NUC	CMB, CYT, MIT.	OTH	N	N	N	N
GRMZM2G449083	LysH-CTLH-CRA-RING uncharacterized protein	Miquel, 2015. Thesis	N	NUC	NUC	NUC	NUC	OTH	N	N	N	N
GRMZM2G132811	ZmCYP33, cyclophilin protein 33	Wang et al., 2017	N	CHL	CHL	CHL	CHL, CYT.	CHLTP	Y	N	N	N
GRMZM2G057374	ZmFKBP17-3, FK506-binding protein 17-3	Yu et al., 2012	N	CHL	CHL	CHL	VAC	CHLTP	Y	N	Y	N
GRMZM2G090689	DnaJ homolog subfamily C member 9 (DNAJC9)	Kanehisa et al. 2016	N	NUC	NUC	NUC	NUC	OTH	N	Y	Y	Y
PLANT CELL REMODELING:												
GRMZM2G000976	CAZy glycosyltransferase 47 family (GT47)	Ekstrom et al., 2014	N	CHL	CHL	SECP	GAP	SP	Y	N	N	N
OTHER GENES:												
GRMZM2G095634	CK1_Casein Kinase_1a.2 - CK1 includes the casein kinase 1 kinases, expressed.	Goodstein et al., 2012	N	CHL	CYT	SECP	CW	OTH	N	N	N	N

GRMZM2G407913	protein SEY1 [EC:3.6.5]	Kanehisa et al., 2016	N	PL	SECP	CYT	CHL	OTH	N	N	N	N
GRMZM2G147014	DEH2, dehydrin protein	Engelberth et al., 2019	N	NUC	NUC	NUC	NUC	OTH	N	Y	N	Y
GRMZM2G035636	E3.4.23.40; phytepsin [EC:3.4.23.40] - Aspartic peptidases	Kanehisa et al., 2016	N	CYT	SECP	SECP	VAC	SP	N	N	N	N
GRMZM2G085711	APY, Apyrase protein	Kanehisa et al., 2016	Y	CYT	SECP	SECP	CMB, CHL, GAP, MIT, NUC	SP	N	N	N	N
GRMZM2G045675	dehydration response related protein, putative, expressed	Goodstein et al., 2012	N	MIT	CHL	NUC	NUC	OTH	Y	N	N	N
GRMZM2G090245	Germin-like protein subfamily 3-member 3	(Mi et al., 2013; Thomas et al., 2003)	Y	CHL	SECP	SECP	CW	SP	N	N	N	N
GRMZM5G836353	Monooxygenase 1	Shi et al., 2019	N	CHL	CHL	CHL	CHL	OTH	N	N	N	N
UNKNOWN:												
GRMZM2G145563	Unknown	Meyer et al., 2017	N	MIT	NUC	NUC	CHL, NUC	OTH	Y	Y	N	Y
GRMZM2G092758	NA	NA	N	CHL	CHL	CHL	NUC	OTH	N	N	N	Y
GRMZM2G517065	NA	NA	N	CHL	SECP	CHL	-	OTH	N	N	N	N
GRMZM2G153635	NA	NA	N	NUC	NUC	NUC	NUC	OTH	N	Y	N	N
GRMZM2G164963	Uncharacterized protein family Ycf49	Jones et al., 2014; Mitchell et al., 2019	N	CHL	CHL	CHL	CMB	CHLTP	Y	Y	Y	Y
GRMZM2G052255	NA	NA	N	CHL	CHL	NUC	NUC	OTH	N	Y	N	N
GRMZM2G029810	NA	NA	N	CHL	CHL	NUC	NUC	OTH	Y	N	N	N
GRMZM5G813709	NA	NA	Y	EXT	SECP	SECP	CHL	SP	N	N	N	N
GRMZM2G056216	NA	NA	N	GAP	SECP	SECP	CMB, CHL, NUC	OTH	Y	N	N	N

ADDITIONAL SUPPLEMENTARY INFORMATION:

These tables are available to download: click here.

Supplementary Table 9: CgEP3 effector responsive genes. This table represents an extension of Supplementary Table 7. Differentially expressed genes (DEGs) from infected maize plants with Δ*CgEP3* strain compared to wild type strain at 24 hours post inoculation. DEGs have been annotated using several databases: BLAST-Koala (KEGG, Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al., 2016), InterPro (P. Jones et al., 2014; Mitchell et al., 2019), PfamScan (Mistry et al., 2007), Panther (Mi et al., 2013; P. D. Thomas et al., 2003) KOG (Bioinformatics et al., 2003), GO (Carbon et al., 2019; Gene & Consortium, 2000) and MaizeGDB (Portwood et al., 2019). Based in Phytozome 10 data of homologous genes that appeared in MaizeGDB database (Goodstein et al., 2012; Portwood et al., 2019). We have found homologous in another plant models as *A. thaliana* and rice.

Supplementary Table 10: Gene ontology (GO) terms of CgEP3 responsive genes. GO terms related to biological process, molecular functions, and cellular components for *C. graminicola* infected leaves at 24 hours post inoculation, performed by the functional enrichment analysis of all the DEGs in web based AgriGO software (Du et al., 2010b; T. Tian et al., 2017). **Supplementary Table 10a and Table 10b,** represent 35 up-regulated DEGs and 21 down-regulated DEGs, respectively.

CHAPTER IV: First report of *Colletotrichum graminicola* causing maize anthracnose in Bosnia and Herzegovina.

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Colletotrichum graminicola is the causal agent of maize (*Zea mays*) anthracnose. The most common symptoms are leaf blight, top dieback and stalk rot (Munkvold and White, 2016), although it can infect all parts of the plant. Due to its negative impact on maize production it is important to study the distribution and propagation of this disease. In September of 2018, maize stem samples exhibiting symptoms of anthracnose stalk rot (glossy, black and irregularity shaped lesions) were collected from a field in the Odžak (Posavska Mahala) region of northeastern Bosnia and Herzegovina. Stem samples, approximately 50mm^2 were dissected and surface-disinfected for 90 seconds in 20% sodium hypochlorite (v/v) and rinsed three times in sterile distilled water. The samples were transferred to one half strength acidified potato dextrose agar (PDA) Petri dishes supplemented with ampicillin (100 µg/mL) and incubated for 4-6 days at 25ºC (Sukno *et al.*, 2008). Single spore isolates from 3 distinct stem pieces were cultured on PDA. The aerial mycelium was dark gray with orange colored spore masses while conidia were falcate, slightly curved, tapered toward the tips and produced in acervuli with setae. The length and width of 50 conidia were measured and ranged from 25 to 35 µm (average 29.8 µm, standard deviation: ± 2.0 µm) and from 7 to 11 µm (average 9.0 µm, standard deviation: ± 0.7 µm), respectively. These morphological characteristics are consistent with *C. graminicola* (Bergstrom and Nicholson, 1999). Two isolates were grown in potato dextrose broth (PDB) for 3 days at 25ºC and total genomic DNA was extracted following the protocol of Baek and Kenerley (1998). The internal transcriber spacer region of rDNA was amplified using universal fungal primers ITS4 and ITS5 and consequently sequenced. GenBank BLAST analysis revealed that the sequences were 100% identical to ITS rDNA sequences of *C. graminicola* isolates. One sequence was deposited in GenBank under accession number MK955539. These results were consistent with the initial identification of *C. graminicola*. To confirm Koch's postulates, maize plants (vegetative stage V3) were placed horizontally in a tray for inoculation and twenty 7.5 µL droplets of a suspension of 3 x 10^5 conidia per milliliter were placed on the surface of the third leaf. The trays were closed to retain moisture and incubated overnight at 23ºC. The next day, the plants were returned to a vertical position and incubated in a growth chamber at 25ºC with 80% humidity and a light cycle of 16h of light and 8h of dark (Vargas *et al.*, 2012). After six days the leaves of the inoculated plants presented brown elongated lesions with chlorotic margins and necrotic centers consistent with *C. graminicola* infection. Controls inoculated with water did not exhibit any symptoms.

Microscopic analysis of the surface of infected leaves revealed acervuli, setae and conidia that were identical to the original isolates. To our knowledge, this is the first report of *Colletotrichum graminicola* causing maize anthracnose in Bosnia and Herzegovina (Arsenijević *et al.,* 1996). Previous reports have shown that maize anthracnose is an important disease in Croatia (Palaversic *et al.,* 2009) and it has been reported in Slovenia. As it may also be a threat for neighboring countries such as Serbia, Romania and Hungary where the cultivation of maize is an important component of the economy, continued surveillance is needed.

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e-Xtra 1: shows leaves of inoculated maize plants.

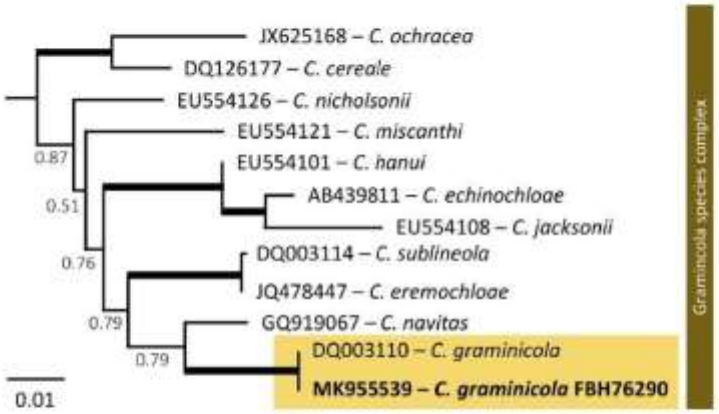
Maize leaves 6 days post-inoculation with conidial suspension from the Bosnia and Herzegovina isolates: FBH76290-4 (A) and FBH76290-7 (B), and mock inoculated control (C). The black dots indicate the inoculation points. The lesions display the typical symptoms of anthracnose leaf blight.

e-Xtra 2: shows phylogenetic tree.

A phylogenetic tree using Bayesian methods was reconstructed using the ITS sequences and those of reference strains belonging to the *Colletotrichum graminicola* species complex. The phylogenetic tree confirmed the identity of the strains isolated from maize as *C. graminicola*.

Maize leaves 6 days post-inoculation with conidial suspension from the Bosnia and Herzegovina isolates: FBH76290-4 (A) and FBH76290-7 (B), and mock inoculated control (C). The black dots indicate the inoculation points. The lesions display the typical symptoms of leaf blight anthracnose.

173x190mm (96 x 96 DPI)



A phylogenetic tree using Bayesian methods was reconstructed using the ITS sequences and those of reference strains belonging to the Colletotrichum graminicola species complex. The phylogenetic tree confirmed the identity of the strains isolated from maize as C. graminicola.

149x100mm (300 x 300 DPI)

CONCLUSIONES

CONCLUSIONES

1. El genoma del hongo *Colletotrichum graminicola* tiene dos genes que codifican las proteínas efectoras que fueron denominadas como *C. graminicola* effector protein 3 (CgEP3) y *C. graminicola* effector protein 6 (CgEP6).

2. El gen *CgEP3* es un efector pequeño que tiene una secuencia de péptido señal que solapa con la secuencia de un posible dominio parcial del tipo de la superfamilia de las nucleósido-fosforilasas. Como consecuencia de una duplicación génica ancestral y su posterior divergencia, el gen *CgEP3* pudo formarse a través de la recombinación ectópica de diferentes partes del genoma. Así, CgEP3 podría haber adquirido una nueva función desde una proteína nucleósido-fosforilasa a la de una proteína efectora.

3. Los análisis *in silico* de la localización subcelular predicen que CgEP3 se encuentra en el núcleo de las células del huésped. La expresión heteróloga y transitoria de *CgEP3* en plantas de tabaco apoyan esta hipótesis, ya que CgEP3 se localiza en el núcleo y citoplasma de las células de las plantas.

4. Gracias a los análisis del perfil transcripcional mediante qRT-PCR, *CgEP3* se expresa en cultivos *in vitro* en las muestras de esporas sin germinar y en micelio crecido saprofíticamente. Asimismo, se expresa durante la etapa biotrófica temprana de la infección de hojas de maíz, teniendo un máximo a las 12 y 24 horas después de la infección. Después los niveles de expresión decrecen rápidamente. Mediante una estirpe que expresa una construcción genética de fusión basada en el promotor nativo de *CgEP3* y GFP, se confirma que CgEP3 se expresa específicamente en las esporas, tubo germinativo y apresorios durante la infección. Por lo que CgEP3 puede ser un efector con función en la pre-penetración de la planta.

5.. Los ensayos de patogenicidad en hojas y tallos de maíz con la estirpe mutante nula de *CgEP3* muestran un tamaño reducido de la lesión, mientras que la reinserción del gen reestablece el fenotipo silvestre. Por lo tanto, CgEP3 está implicado en la virulencia de *C. graminicola.* Las plantas infectadas con la estirpe que ha sido transformada con la complementación parcial del gen no recuperan el fenotipo inicial, por lo que se sugiere que CgEP3 necesita la secuencia completa de la proteína para que se reestablezca su función. La expresión constitutiva de *CgEP3* no tiene un efecto en la virulencia.

6. Mediante ensayos de cuantificación de biomasa fúngica relativa y penetración durante la infección, se demuestra que CgEP3 es necesario para la penetración de la epidermis de la planta y la colonización del huésped.

7. Los análisis transcriptómicos a través de RNA-Seq de infecciones de hojas de maíz a 24 horas después de la infección revelan 56 genes diferencialmente expresados (DEGs) en plantas infectadas con la estirpe mutante de *CgEP3*. Estos genes que responden al efector representan mecanismos de defensa independientes que pueden ser suprimidos o inducidos por CgEP3 durante la etapa temprana de la infección de las hojas del maíz. CgEP3 podría contrarrestar algunos de los mecanismos de defensa de pre-invasión de la planta y así la colonización se daría con el menor esfuerzo.

8. CgEP6 es un efector que tiene cuatro dominios ricos en extensinas (PRICHEXTENSN) en su estructura. También tiene cinco motivos “poliprolinas” tres de los cuales podrían interaccionar con el ADN/ARN. Esta proteína tiene un alto contenido en prolinas (22%).

9. Los análisis filogenéticos demuestran que CgEP6 está conservado en el género *Colletotrichum,* ya que se han encontrado 21 proteínas homólogas a CgEP6 en siete de los complejos de especies. A su vez, se identificaron cuatro alelos de CgEP6 en *C. graminicola* que variaban en tamaño y porcentaje de prolinas.

10. Los análisis *in silico* de la localización subcelular predicen que CgEP6 tiene localización nuclear. Los experimentos de expresión heteróloga y transitoria de *CgEP6* en plantas de tabaco apoyan la anterior predicción ya que CgEP6 se localiza en el núcleo y citoplasma de las células vegetales.

11. El perfil transcriptómico de *CgEP6* determina que se expresa durante la etapa biotrófica temprana de la infección, teniendo un máximo de expresión a las 12 y 24 horas después de la infección siendo aun detectable 36 y 48 después de la infección.

12. Las infecciones de hojas con la estirpe mutante de *CgEP6* demuestran que tiene un tamaño de la lesión menor comparada con las hojas infectadas con la estirpe silvestre a 3 y 4 días después de la inoculación. Por lo que CgEP6 tiene un papel en la virulencia de *C. graminicola* durante el desarrollo de la antracnosis del maíz.

13. Los ensayos de fenotipado en diferentes medios de cultivo demuestran que la falta de *CgEP6* produce que *C. graminicola* sea más sensible frente a estreses de la pared celular como el rojo Congo, por lo que la integridad de la pared celular puede estar afectada.

14. Tras el aislado de diferentes estirpes fúngicas a partir de muestras de tallos infectados de campos de maíz de Bosnia y Herzegovina, se identifica la especie *C. graminicola* a través estudios morfológicos, filogenéticos y de patogénesis. Esta es la primera descripción de *C. graminicola* en Bosnia y Herzegovina.

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