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CARACTERIZACIÓN BIOQUÍMICO-MOLECULAR DE LA ENDO-β-(1,6)-D-GALACTANASA Y LA α-L-ARABINOFURANOSIDASA DE DOS RAZAS DE *Colletotrichum lindemuthianum* CON DISTINTO ESTILO DE VIDA

TESIS PARA OBTENER EL GRADO DE DOCTORA EN CIENCIAS BIOLÓGICAS
QUE PRESENTA

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I. RESUMEN GENERAL

Colletotrichum lindemuthianum es el causante de la antracnosis en plantas de frijol (*Phaseolus vulgaris*). Entre de las enzimas que el hongo libera para la hidrólisis de la pared celular vegetal (PCV) se encuentran la endo-β-(1,6)-D-galactanasa (EBG) y la α-L-arabinofuranosidasa (ABF) las cuales intervienen en la degradación de arabinogalactanos tipo II y hemicelulosas. En el presente trabajo se realizó el aislamiento y la caracterización molecular de los genes de ambas enzimas en dos razas de *C. lindemuthianum* con diferentes grados de patogenicidad. Tanto la secuencia de ADN y cDNA que codifican para la endo-β-(1,6)-D-galactanasa poseen 1440 pb con un UTR en el extremo 3' de 118 pb y 37 pb respectivamente. Los genes *ebg* de la raza patógena y no patógena de *C. lindemuthianum* presentaron 100% de indentidad, mientras que al realizar una comparación con genes de EBG identificados en otras especies de *Colletotrichum* se obtuvieron valores de 79 a 94% de identidad y 88-97% de similitud. La secuencia deducida a partir del gen *ebg* fue de 480 aminoácidos con un péptido señal de 20 residuos. En la secuencia de aminoácidos se identificó el dominio característico de la familia 30 de las glicosil hidrolasas y el análisis 3D corroboró la estructura típica de esta familia. La secuencia codificante del gen de la EBG A consta de 2231 pb, con extremos UTR 5' y 3' de 20 y 30 pb, respectivamente. En ésta, se identificaron cinco exones separados por cuatro intrones. Los genes *abfA* en las razas patógena y no patógena presentaron 100% de indentidad. La comparación con bases de datos mostró 85-98% de identidad y 90 a 98% de similitud con genes codificantes de ABF en otras especies de *Colletotrichum*. La secuencia codificante del gen *abfA* consta de 1995 pb y una secuencia deducida de 665 aminoácidos con un péptido señal de 17 residuos; además, se identificó el dominio característico de la familia 51 de las glicosil hidrolasas y el análisis 3D corroboró la estructura típica de esta familia. Los resultados del análisis de expresión mostraron represión catabólica por glucosa, indicando que en la raza patógena los productos de degradación de la arabinogalactana, xilana y otros componentes de la PCV pueden inducir de manera coordinada altos niveles de expresion de *ebg* y *abfA*. En la raza no patógena, la arabinogalactana y la xilana indujeron bajos niveles de expression de *ebg* y solo la arabinogalactana indujo altos niveles de expresion de *abfA*. Cuando se analizó la actividad de arabinofuranosidasa secretada en diferentes tiempos de cultivo, aunque ambas razas mostraron niveles de actividad enzimática en arabinogalactana, xilana y PCV, la raza no patógena mostró los niveles máximos en micelio

inducido con xilana. En la región regulatoria de ambos genes se identificaron varios elementos que pueden ejercer una activación transcripcional diferencial en los promotores basales. Adicionalmente, se identificaron secuencias putativas de unión a ADN para factores de transcripción que regulan la expresión de xilanasas y celulasas. Se estableció que los genes de *egb* y *abfA* en las especies de *Colletotrichum* analizadas, con pocas excepciones, no han sufrido duplicaciones. El análisis filogenético de EBGs y ABFAs de especies de *Colletotrichum* corroboró que la especie hermana de *C. lidemuthianum* es *C. orbiculare*. Aunque se identificaron clados de especies estrechamente relacionadas, la topología del árbol filogenético de EBG y el de ABFA en las especies analizadas muestran historias evolutivas diferentes. Por último, se realizó la purificación de la enzima ABF a partir de cultivos de la raza patógena crecida con arabinogalactana como única fuente de carbono. La proteína fue purificada 91.8 veces y mostró un tamaño aproximado de 82 kDa, pH y temperatura óptimos de 6.0 y 50ºC, respectivamente. La actividad de ABF incrementó al adicionar Mg^{2+}, Mn^{2+} y Ca^{2+}. Finalmente, se calcularon las constantes cinéticas para la proteína obteniendo una K_m de 0.23 mM y una V_{max} de 303.03 nM de 4MU/min/µg de proteína. Gracias a sus propiedades bioquímicas, la ABF purificada podría considerarse una buena candidata para ser utilizada en procesos industriales y biotecnológicos.

Palabras clave: *Colletotrichum lindemuthianum*, *Phaseolus vulgaris*, endo-β-(1,6)-D-galactanasa, α-L-arabinofuranosidasa, arabinogalactano.

II. SUMMARY

Colletotrichum lindemuthianum is the etiological agent of antrachnose in common bean (*Phaseolus vulgaris*). Endo-β-(1,6)-D-galactanase (EBG) and α-L-arabinofuranosidase (ABF) are polysaccharidases secreted by *C. lindemuthianum* that degrade plant cell walls (PCW), specifically arabinogalactan type II and hemicelluloses. Here, we report the isolation and molecular characterization of the genes encoding for EBG and ABF in both pathogenic and non-pathogenic races of *C. lindemuthianum*. DNA and cDNA encoding endo-β-(1,6)-D-galactanase are 1440 bp in length with a 3'UTR of 118 bp and 37 pb respectively. The DNA and cDNA sequences from both races showed 100% identity. Comparison with corresponding sequences in GenBank revealed 79-94% identity and 88-97% similarity with EBG genes from other *Colletotrichum* species. The mature protein is 480 amino acids in length with a peptide signal of 20 residues. The characteristic domain of GH30 proeins was located in the amino acid sequence and 3D analysis corroborated the tipical structure of members of this family. DNA encoding for ABF A is 2231 bp in length with a 3' and 5'UTR of 20 and 30 bp, respectively. The presence of five exons separated by four introns was also located. The DNA sequences from both races showed 100% identity. Comparison with corresponding sequences in GenBank revealed 85-98% identity and 90-98% similarity with ABF genes from other *Colletotrichum* species. Prediction of *abfA* coding sequence showed an ORF of 1995 bp. The mature protein is 665 amino acids in length with a peptide signal of 17 residues. The characteristic domain of GH51 proteins was located in the amino acid sequence and 3D analysis corroborated the tipical structure of this family. Results of expression analyses showed a catabolic repression by glucose and indicate that in the pathogenic race the degradation products of arabinogalactan, xylan and other plant cell wall components can induce a coordinated and high expression of *ebg* and *abfA*. In the non-pathogenic race, arabinogalactan and xylan induce low levels of *ebg* expression and only arabinogalactan slightly induced high levels of *abfA* expression. When the arabinofuranosidase activity secreted at different periods of growth was measured, although both races showed levels of enzymatic activity in arabinogalactan, xylan and PCW, the non-pathogenic race showed maximum levels in mycelium induced with xylan. several elements were identified in the regulatory region of both genes that can exert a differential transcriptional activation in the basal promoters. Additionally, putative DNA-binding sequences for transcription factors that regulate

the expression of xylanases and cellulases were identified. It was established that, with few exceptions, the genes of *egb* and *abfA* in the *Colletotrichum* species analyzed have not undergone duplications. Phylogenetic analysis of EBGs and ABFAs of *Colletotrichum* species showed that *C. lidemuthianum* is the sister species of *C. orbiculare*. Although clades of closely related species were identified, the topology of the EBG phylogenetic tree and that of ABFA in the species analyzed show different evolutionary histories. Finally, ABF was purified from the pathogenic race grown in media supplemented with arabinogalactan as the sole carbon source. ABF was purified 91.8-fold with a specific activity of 331.6 nM of 4-MU/min/μg of mycelium protein. The enzyme molecular mass and optima pH and temperature were 82 kDa, 6.0 and 50ºC, respectively. Apparent K_m and V_{max} values were 0.23 mM and 303.03 nM of 4MU/min/μg of mycelium protein, respectively. The rather wide range of pH and temperature, as well as the high substrate specificity, make ABF from *C. lindemuthianum* a good candidate to be used in biotechnological and industrial processes.

Key words: *Colletotrichum lindemuthianum*, *Phaseolus vulgaris*, endo-β-(1,6)-D-galactanase, α-L-arabinofuranosidase, arabinogalactan.

III. INTRODUCCIÓN GENERAL

1. Diversidad de patotipos de *Colletotrichum lindemuthianum*

C. lindemuthianum (Sacc. & Magnus) Briosi & Cavara (Ascomicota, Sordariomicetes, Glomeraceae) es un hongo patógeno que causa la enfermedad de la antracnosis en plantas del frijol común *Phaseolus vulgaris* (Pastor-Corrales & Erazo, 1994).

C. lindemuthianum presenta distintas cepas patogénicas, clasificadas como razas fisiológicas o patotipos, que infectan diferencialmente a un grupo de cultivares de una misma especie hospedera. El sistema de denominación de patotipos en *C. lindemuthianum* se realizó en base a la interacción de distintos aislados del hongo con doce cultivares diferenciales de *Phaseolus vulgaris*. Cada diferencial posee un valor de 2^n, donde "n" toma el valor de 0 a 11. Cuando un aislado es capaz de establecer una interacción de compatibilidad con uno o más cultivares, el aislado será denominado en base a la suma de los valores asignados de los cultivares que fueron susceptibles al aislado probado. Si algún aislado es incapaz de establecer una interacción de compatibilidad con los cultivares diferenciales, este será designado como patotipo cero (Rodríguez-Guerra et al., 2006).

En México, se identificaron 56 patotipos de los más de 100 reportados a nivel mundial (Sánchez-García et al., 2009). Nuestro grupo de trabajo cuenta con dos patotipos de *C. lindemuthianum* con diferente grado de patogenicidad: la raza 0 (no patógena) y la 1472 (patógena). De la raza 0 se obtuvieron 30 aislados provenientes de los estados de Durango, Jalisco, Michoacán, México, Tlaxcala y Morelos. Por otra parte, dentro de los patotipos más agresivos que se aislaron en el país, la raza 1472 fue las más frecuentemente encontrada con un total de 6 aislados procedentes de los estados de Zacatecas, Guerrero, Hidalgo, México, y Chiapas (Rodríguez-Guerra et al., 2006).

1.1. Estrategia de nutrición/infección de *C. lindemuthinum*

C. lindemuthianum utiliza como estrategia de nutrición/infección la hemibiotrofía. Ésta consta de una fase biotrófica donde los nutrientes se obtienen de células vivas y una fase necrotrófica caracterizada por el desarrollo de hifas secundarias que se ramifican a través de los tejidos del huésped provocando la muerte de las células (Perfect, 1999; 2001).

Durante la fase biotrófica, una espora del hongo se adhiere a la superficie de la planta. Esta adhesión es posible gracias que ésta posee una cubierta fibrilar rica en carbohidratos con un número irregular de poros en su superficie. La cubierta de la espora de *C. lindemuthianum* le permite adherirse a superficies hidrofóbicas de la planta y es la superficie de contacto requerida para iniciar la diferenciación del tubo germinal y del apresorio (Rawlings, 2007). Se ha reportado en varias especies de *Colletotrichum* que las esporas sensan señales físicas y químicas de la superficie de la planta capaces de disparar la germinación y la formación de un tubo germinal pequeño que posteriormente se diferencia en un apresorio (4-5 μm) inicialmente hialino pero que rápidamente se melaniza. El apresorio crea una alta presión de turgencia que proporciona la fuerza mecánica para la perforación de la cutícula y la penetración de la pared celular (PC) de las células de la epidermis. Estas hifas de penetración se ensanchan dentro de las células epidermales formando vesículas de infección e hifas primarias intracelulares, las cuales colonizan algunas células del hospedero realizando su recorrido a través del lúmen celular causando un daño mínimo a las PC´s. Tanto las vesículas de infección como las hifas primarias se encuentran rodeadas por una matriz extracitoplásmica de glicoproteínas secretadas que está conectada con los protoplastos vegetales y que separa la PC del hongo de la membrana plasmática de las células del huésped (Idnurm & Howlett, 2001; Mendgen, 2002; O´Conell, 1985).

Entre las 48 y 72 horas post inoculación, dependiendo de las condiciones ambientales, comienza la fase necrotrófica. Esta fase se caracteriza por el desarrollo de hifas secundarias que crecen tanto intra- como intercelularmente y se ramifican dentro de los tejidos de la planta, son de un diámetro más pequeño que las hifas primarias y carecen de cubierta extracitoplasmática. Además, las hifas secundarias producen una gran cantidad de toxinas y enzimas hidrolíticas capaces de degradar la PC y proteínas causando lisis y muerte celular al penetrar los protoplastos. Es durante éste periodo cuando se manifiestan los síntomas de antracnosis en la planta (Divon, 2007; Mendgen, 2002; Münch, 2008; O´Conell, 1985).

Las enzimas hidrolíticas secretadas por las hifas secundarias durante el periodo necrotrófico son fundamentales debido a que la despolimerización de la PC permite la liberación de una amplia variedad de azúcares monoméricos y oligoméricos que representan una fuente directa de carbono que provee de nutrientes al hongo (Münch, 2008). En un estudio previo realizado por Wijensudera y col. (1989), se encontró que en cultivos de *C. lindemuthianum* se produce la

secreción de varias enzimas, principalmente una endo-poligalacturonasa, dos pectin liasas, α y β-galactopiranosidasas, α-arabinofuranosidasa y proteasas durante la degradación de la PC.

Adicionalmente, Dufresne y col. (2000) caracterizaron el gen *CLTA1* (*C. lindemuthianum transcriptional activator 1*) que codifica para un activador transcripcional putativo del tipo dedo de zinc que podría estar involucrado en la transición entre la fase biotrófica y la necrotrófica. Demostraron que en plantas con silenciamiento de este gen, el hongo era capaz de desarrollar hifas primarias pero no hifas secundarias. Fenotípicamente se observó solo la aparición de lesiones necróticas típicas de una respuesta hipersensible (Dufresne, 2000).

2. Estructura de la PCV

La PCV está formada por microfibrillas de celulosa, un polisacárido constituido por moléculas de glucosa unidas por enlaces β-(1-4) formando una red cristalina que se encuentra inmersa en una matriz de polisacáridos de dos tipos: polisacáridos pécticos que incluyen homogalacturonano, xilogalacturonano, ramnogalacturonano, entre otros polisacáridos (Harholt, 2010) y la hemicelulosa, un grupo heterogéneo de polisacáridos caracterizados por poseer moléculas de glucosa, manosa o xilosa unidos por enlaces β-(1-4). Los polisacáridos más importantes en la hemicelulosa son xiloglucanos, xilana, manana y glucomanana de los cuales el xiloglucano es el más abundante de las hemicelulosas. En las paredes primarias también se han encontrado en menor cantidad algunos otros polisacáridos tales como galactanos, arabinanos y arabinogalactanos (Scheller, 2010).

La pectina está constituída principalmente por homogalacturonano, xilogalacturonano, apigalacturonano y ramnogalacturonano. La proporción entre estos polisacáridos es variable pero típicamente el homogalacturonano es el polisacárido más abundante constituyendo aproxiamadamente el 65% de la pectina (Lara-Márquez et al., 2011; Harholt et al., 2010)

2.1 Arabinogalactanos (AGs)

Asociados a hemicelulosas y pectina, se encuentran los arabinogalactanos (AGs). De acuerdo al tipo de enlaces que presentan, los AGs han sido clasificados en arabinogalactanos tipo I y II. Los arabinogalactanos tipo I se caracterizan por presentar una cadena lineal de residuos de galactopiranosa unidos por enlaces β-(1,4) sustituídos por cadenas de α-(1,3)-arabinofuranosa, (Clarke et al., 1979; Sakamoto & Ishimaru, 2013), mientras que los AGs de tipo II consisten en una cadena de residuos de D-galactosa unidos por enlaces β-(1,3), sustituidos en C(*O*)6 por

cadenas laterales de β-(1,6)-D-galactopiranosa con terminales no reductores de arabinopiranosa ó arabinofuranosa, los cuales se asocian a la β-(1,6)-galactosa por enlaces α-(1-3) y α-(1,5) (Gaspar et al., 2001; Showalter, 2001).

Se ha reportado que algunos AGs tipo II pueden estar modificados en menor cantidad por otros monosacáridos como son L-ramnosa, D-manosa, D-xilosa, D-glucosa, L-fucosa, D-glucosamina, ácido D-glucurónico y ácido D-galacturónico (Fig. 1) (Showalter, 2001).

Mediante resonancia magnética nuclear (RMN) se ha establecido que la estructura molecular de los AGs presenta tres características comunes: una cadena principal de galactanos compuesta por dos bloques de β-(1,3)-trigalactosil unidos por un enlace β-(1,6), cadenas laterales bifurcadas con arabinosa, ramnosa, ácido glucurónico y β-(1,6)-galactosa unida a la galactosa 1 y 2 de la cadena principal de trigalactosil repetidos y, por último, una cadena lateral común compuesta por hasta seis residuos conteniendo α-L-arabinofuranosa-(1,5)-α-L-arabinofuranosa-(1,3)-L-arabinofuranosa-(1,3) y α-L-ramnosa- (1,4)-β-D-ácido glucurónico-(1,6), ambos unidos a galactosa (Tan et al., 2010).
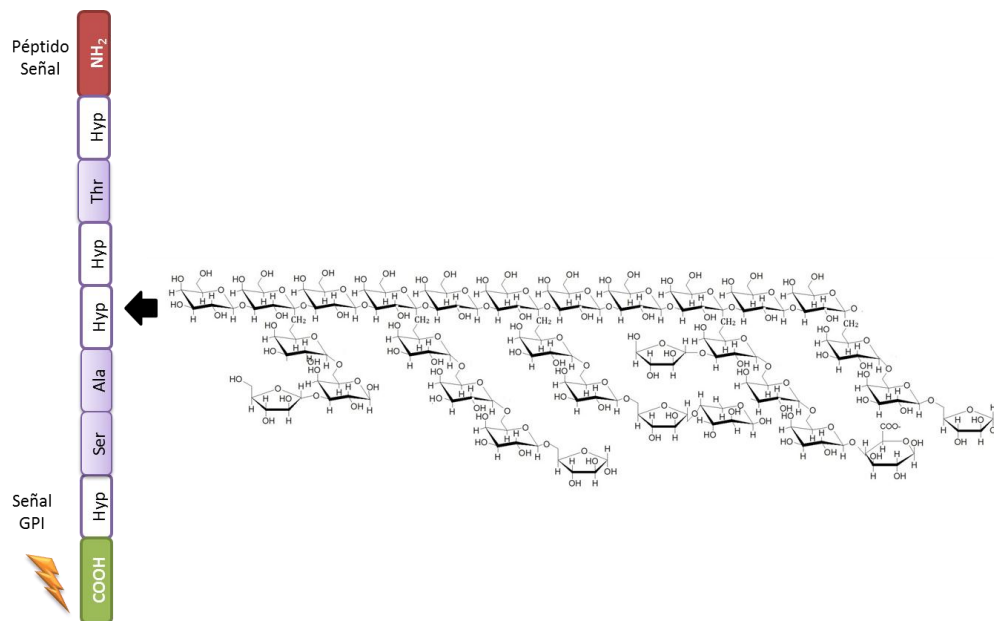

Figura 1. Estructura de una proteína arabinogalactano.

Los AGs pueden estar unidos a un núcleo proteico formando las denominadas proteínas arabinogalactano (AGPs; Fig. 1). Las AGPs son glicorproteínas constituidas por menos del 10% de proteína que se encuentra O-glicosilada por uno o más complejos de arabinogalactanos tipo II

lo que constituye el 90% de la AGP y se pueden ubicar en la membrana plasmática, PC, en el espacio apoplástico y en secreciones (superficie del estigma y exudados) (Ellis et al., 2010).

Los AGs tipo II se encuentran unidos al núcleo protéico vía hidroxiprolina y posiblemente a través de serina y treonina o por medio de residuos de arabinosa unidos a hidroxiprolina; en algunos casos la región de la proteína rica en hidroxiprolina se encuentra interrumpida por una pequeña región básica. Además de éste dominio rico en hidroxiprolina, el núcleo proteico se caracteriza por tener un dominio C-terminal hidrofóbico (Fig. 1) (Gaspar et al., 2001; Showalter, 2001).

Las AGPs pueden estar asociadas a la membrana plasmática a través de anclas de glicosil fosfatidil inositol (GPI), donde el C-terminal del dominio hidrofóbico es la señal para la adición del GPI el cual se acopla vía fosfoetanolamina al oligosacárido D-Manosa-(1-2)-α-D-Manosa-(1,6)-α-D-Manosa (1-4)-α-D-N-acetilglucosamina unido a un residuo de inositilfosfoceramida (Oxley & Bacic, 1999).

Estas proteínas se han dividido en dos clases en función de los aminoácidos que componen su núcleo proteico: AGPs clásicas caracterizadas por poseer un núcleo proteico rico en hidroxiprolina, alanina, serina, treonina y glicina, clasificadas también como una de las tres familias de proteínas ricas en hidroxiprolina (HGRPs) (Showalter et al., 2010), y AGPs no clásicas las cuales poseen un núcleo protéico pobre en hidroxiprolina pero rico en cisteína o asparagina. El análisis de diferentes tejidos y de varias especies de plantas sugiere que hay gran heterogeneidad tanto en el componente de carbohidrato como en el de proteína (Gaspar et al., 2001; Showalter, 2001).

3. Degradación enzimática de AGs

Como parte del conjunto de enzimas que poseen los hongos filamentosos para degradar la PCV se encuentran las enzimas encargadas de la degradación de AGs tipo II. Se han descrito hasta el momento enzimas principales y accesorias encargadas de la hidrólisis enzimática de este polisacárido.

3.1 Enzimas principales

Las endo-β-(1,3)-galactanasas (EC 3.2.1.181; familia GH 16) y las exo-β-(1,3)-galactanasas (EC 3.2.1.145; familia GH 43) llevan a cabo la degradación enzimática de la cadena principal de β-

(1,3)-galactosa. La endo-β-(1,3)-galactanasa hidroliza β-(1,3)-galactanos y β-(1,3)-galacto-oligosacáridos con grado de polimerización mayor a tres (Kotake et al., 2011), mientras que la exo-β-(1,3)-galactanasa libera residuos monoméricos de galactosa de los extremos no reductores de la cadena de β-(1,3)-galactosa incrementando su actividad cuando existe un mayor grado de polimerización de la cadena (Fig. 2) (Tsumuraya et al., 1990; Okawa et al., 2013) (Fig. 2).

La β-galactosidasa del hongo *Hypocrea jecorina* cataliza la hidrólisis de extremos no reductores de β-D-galactosa de AGs tipo II pero es inhibida por su producto de reacción (β-galactosa). Esta enzima ha sido probada principalmente en disacáridos como la lactosa (Gamauf et al., 2007).
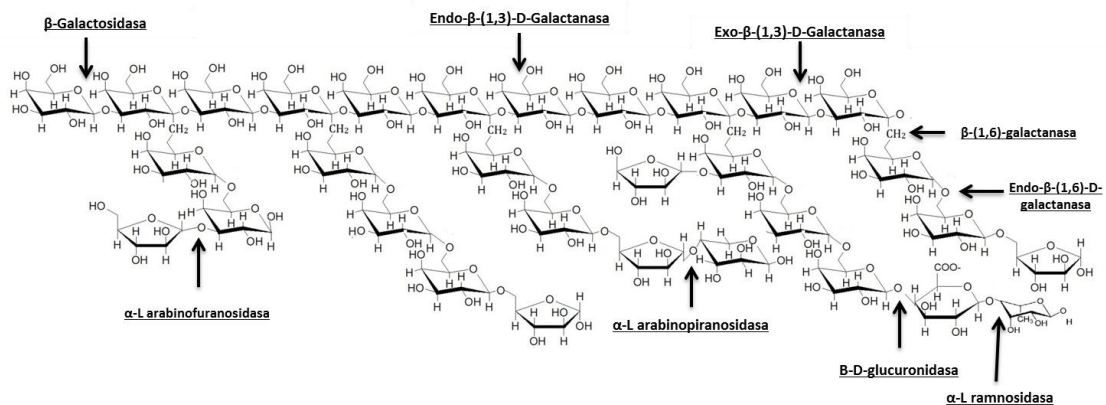
Figura 2. Degradación enzimática de AGs tipo II.

3.2. Enzimas accesorias

3.2.1. Endo-β-(1,6)-galactanasas (EBGs)

Las endo-β-(1,6)-galactanasas (EC 3.2.1.164) se encuentran tanto en bacterias como en hongos y están encargadas de la hidrólisis de enlaces β-(1,6)-galactosídicos de AGs tipo II o de metil-β-(1,6)-galactohexosas liberando una mezcla de galacto-oligosacáridos con grado de polimerización variable, los cuales pueden contener β-(1,6)-galactobiosa y una pequeña cantidad de galactosa (Brillouet et al., 1991; Okemoto et al., 2003; Sakamoto & Ishimaru, 2013).

Reportes recientes apuntan a la existencia de dos enzimas diferentes capaces de degradar enlaces β-(1,6)-galactosídicos: la endo-β-(1,6)-galactanasa y la β-(1,6)-galactanasa. La primera de estas enzimas hidroliza específicamente β-(1,6)-galactooligosacáridos con grado de polimerización mayor a tres, liberando galactoligosacáridos dos a cinco residuos por lo que es clasificada como

endo-galactanasa (Okemoto et al., 2003). En cambio, la β-(1,6)-galactanasa no es capaz de hidrolizar galactooligosacáridos de más de dos residuos, actuando específicamente sobre galactobiosa (Sakamoto et al., 2007; Sakamoto & Ishimaru, 2013).

La actividad específica de endo-β-(1,6)-galactanasa se reportó en 1991 cuando se purificó la enzima por primera vez a partir de un extracto crudo de *Aspergillus niger* y se describió su actividad sobre AGs de uva, mientras que la actividad de β-(1,6)-galactanasa se evaluó en el 2003 cuando se purificó a partir de preparaciones comerciales de pectinasa producidas por *A. niger* y *A. aculeatus* (Brillouet et al., 1991; Luonteri et al., 2003).

Respecto al gen que codifica para la endo-β-(1,6)-galactanasa se conoce que al menos en *Trichoderma viride* hay solo uno (Kotake et al., 2004). Por otro lado, se ha caracterizado el cDNA codificante a partir de los hongos *T. viride* y *Neurospora crassa* así como del actinomiceto *Streptomyces avermitilis* (Tabla 1) (Ichinose et al., 2008). La secuencia de aminoácidos deducidos de la enzima presenta bajos porcentajes de similitud con otras glicosil hidrolasas conocidas. En un principio, se sugirió que esta enzima podría ser un miembro distante de la familia 5 de las glicosil hidrolasas (GH5), debido a que la predicción de la estructura tridimensional de la secuencia de *T. viride* mostraba alta similitud con la β-mananasa de *T. ressei* perteneciente a la familia GH5. Sin embargo, recientemente se le ha clasificado dentro de la familia GH30 (Sakamoto & Ishimaru, 2013). Esta familia pertenece al clan A y agrupa enzimas como endo-β-1,4-xilanasas (EC 3.2.1.8), β-glucosidasas (3.2.1.21), β-xilosidasas (EC 3.2.1.37) y fucosidasas (EC 3.2.1.38), entre otras. Estas enzimas se caracterizan por tener estructuras $(\beta/\alpha)_8$, operan por el mecanismo de retención vía doble desplazamiento y sus sitios catalíticos son dos residuos de ácido glutámico que actúan como par ácido/base y un nucleófilo (Kotake et al., 2004). Adicionalmente, se tienen reportes de la expresión heteróloga de secuencias codificantes de endo-β-(1,6)-galactanasas tanto en *Escherichia coli* como en *Pichia pastoris* (Tabla 1 y 2).

Tabla 1. Genes de endo-β-(1,6)-D-galactanasas reportados.

Especie	Nucleótidos	Aminoácidos deducidos	No. copias	*N*-glyc	Expresión heteróloga	Residuos catalíticos	No. de acceso	Referencia
Trichoderma viride	1447	479	1	3	*Escherichia coli*	Glu/Glu	AB104898	(Kotake et al., 2004)
Neurospora crassa	1464	488	ND	ND	*Pichia pastoris*	ND	NCU09702	(Takata et al., 2010)
Streptomyces avermitilis	1476	491	ND	ND	*Escherichia coli*	ND	BAC72917	(Ichinose et al., 2008)

ND: no determinado

Por otro lado, se cuenta con dos reportes de la purificación y caracterización bioquímica de endo-β-(1,6)-galactanasas nativas, procedentes de *A. niger* y *T. viride* y dos proteínas recombinantes (Tabla 2).

Tabla 2. Propiedades bioquímicas de endo-β-(1,6)-D-galactanasas purificadas.

Especie	Peso molecular (KDa)	Punto soeléctrico	pH óptimo	Temperatura óptima (ºC)	Constantes cinéticas		Referencia
					K_m	V_{max}	
Aspergillus niger	60[a]	ND	3.5	60	ND	ND	(Brillouet et al., 1991)
Trichoderma viride	47[a]	5.4	4.3	30	ND	ND	(Okemoto et al., 2003)
Neurospora crassa[c]	54[a]/51.263[b]	ND	5.5	50	ND	ND	(Takata et al., 2010)
Streptomyces avermitilis[c]	52[a]	ND	5.5	40	ND	ND	(Ichinose et al., 2008)

ND: no determinado
a: Datos de la enzima por SDS-PAGE
b: Valor calculado de la proteína madura
c: Proteína recombinante

3.2.2. α-L-arabinofuranosidasas (ABFs)

Las α-L-arabinofuranosidasas, (E:C 3.2.1.55) catalizan la hidrólisis de extremos no reductores de arabinosa unidos por enlaces α-L-(1,2), α-L-(1,3) y α-L-(1,5) de diferentes oligosacáridos. Son enzimas extracelulares que han sido purificadas de bacterias, plantas y hongos (Numan & Bhoshe, 2006).

Durante la década de los 90, se reportó la purificación de varias arabinofuranosidasas a partir de distintas especies de hongos y bacterias. Sin embargo, no en todos los casos se evaluó la actividad catalítica de las mismas sobre arabinogalactano. De acuerdo a su secuencia de aminoácidos, similitudes estructurales y especificidad de sustrato, estas enzimas se han clasificado dentro de las familias 2, 3, 10, 43, 51, 54 y 62 de las glicosil hidrolasas . De las α-L-arabinofuranosidasas purificadas capaces de degradadar AGs, se han descrito dos grupos. El primero de ellos, perteneciente a la familia GH51, es capaz de hidrolizar pequeños sustratos incluyendo cadenas cortas de arabinooligosacáridos, mientras que el segundo grupo, de la familia GH54, es capaz de hidroliza, sustratos poliméricos como arabinoxilanos y arabinogalactanos además de arabinooligosacáridos. (De Ioannes et al., 2000). Además de los reportes bioquímicos de α-L-arabinofuranosidasas (Tabla 4), se cuenta también con varios ADNc secuenciados (Tabla 3).

Tabla 3. Genes codificantes de la α-L-arabinofuranosidasas de la GH51 en hongos

Especie	Nucleótidos (pb)	Aminoácidos deducidos	No. copias	Glicosilación	Expresión heteróloga	Residuos catalíticos	No. de acceso	Referencia
Aspergillus niger N402	2237[a] 1884[b]	628 603[c]	ND	*N*-glic (10)	ND	ND	L29005.1	(Flipphi et al., 1994; Flipphi et al., 1993)
Aspergillus kawachii	1874[b]	628	ND	*N*-glic (9)	ND	Glu 368/Glu 449	AB085903.1	(Koseki et al., 2003)
Aspergillus awamori	1874[b]	628	ND	*N*-glic (9)	ND	Glu 368/Glu 449	AB046702.2	(Koseki et al., 2003)
Aspergillus oryzae	1443[b]	481	1	ND	Sobre expresión	Glu184/Glu 310	BD143578.1	(Matsumura et al., 2004)
Penicillium purpurogenum	1908[b]	635 618[c]	ND	*N*-glic (359, 626)	ND	Glu 357/Glu434	EF490448	(Fritz et al., 2008)
Aspergillus niger ATCC 120120	2239[a] 1887[b]	628 603[c]	ND	*N*-glic (10)	*P. pastoris*	ND	HM004501.1	(Alias et al., 2011)
Pleorotus ostreatus	4061[a] 1941[b]	646 626[c]	ND	*O*-glic (Ser 160)	*P. pastoris* y *Kluyveromyces lactis*	Glu 364/Glu 471	HE565355.1[a] HE565356.1[b]	(Amore et al., 2012)
Penicillium chrysogenum 31B	2272[a] 1905[b]	635	ND	*N*-glic (7)	*E. coli*	ND	AB506805.1[a] AB461442[b]	(Sakamoto et al., 2013)

ND: no determinado
a: ADN genómico
b: cDNA
c: Proteína madura

A diferencia de las galactanasas, donde se ha encontrado un solo gen codificante de las enzimas, en el caso de la α-L-arabinofuranosidasas se reportaron hasta cuatro genes codificantes en *Penicillium foniculosum* (Guais et al., 2010). De la misma forma que para las endo-β-(1,6)-D-galactanasas, se cuenta con varios reportes de la expresión heteróloga de la α-L-arabinofuranosidasas en *P. pastoris* (Tabla 3).

Tabla 4. Propiedades bioquímicas de ABFs GH51 caracterizadas

Especie	Peso molecular (KDa)	Punto isoeléctrico	pH óptimo	Temperatura óptima (ºC)	Constantes cinéticas		Referencia
					K_m	V_{max}	
Aspergillus niger	128[a]	6	4.1	50	0.16 g/l	15700 min⁻¹	(Rombouts et al., 1988)
Aspergillus niger N402	83[a]/68[b] 65.4[c]	3.3	3.4	46	6.8x10^{4} M	33 I. U.	(Flipphi et al., 1994; vd Veen et al., 1991)
Aspergillus awamori IFO 4033	81[a]	3.3	4	60	ND	ND	(Kaneko et al., 1998)
Penicillium capsulatum	64.5[a]	4.15	4	60	0.18 mM	ND	(Filho et al., 1996)
Aspergillus kawachii	80[a]	ND	4	55	ND	ND	(Koseki et al., 2003) Koseki, 2003
Penicillium chrysogenum	79[a]/75[b] 71[c]	ND	5	50	1.32 mM	ND	(Sakamoto & Ishimaru, 2013)
A. orizae[d]	55[a]/54[c] 228[c]	4.6	5	50	ND	ND	(Matsumura et al., 2004)
Penicilium	70[a]/56[c]	5.3	5	60	0.098	ND	(Fritz et al., 2008)

purpurogenum	67.212[c]					mM	
Aspergillus niger ATCC 120120[d]	83[a]/66[b] 65.334[c]	ND	4	50	0.93 mM	17.86 μMol/ml/min	(Alias et al., 2011)
Penicillium ostreatus[d]	81.5[a]/39[e] 68.912[c]	ND	5	40	0.64 mM	3010 min^{-1}	(Amore et al., 2012)
Penicillium chrysogenum 31B[d]	85[a] 67.497[c]	ND	5	50	ND	ND	(Sakamoto et al., 2013)

ND: no determinado
a: Datos de la enzima por SDS-PAGE
b: Datos de la enzima desglicosilada por SDS-PAGE
c: Valor calculado de la proteína madura
d: Proteína recombinante
e: Valor calculado por filtración en gel

Respecto a las arabinopiranosidasas, se purificaron dos enzimas bifuncionales con actividades de β-L-arabinopiranosidasa/α-D-galactopiranosidasa a partir de cultivos de *F. oxisporum* 12S llamadas Fo/AP1 y Fo/AP2. Ambas enzimas hidrolizan arabinogalactana de madera de alerce (LWAG) liberando arabinosa pero no galactomananos presentes en las cadenas laterales de α-D-galactopiranosil. La β-glucuronidasa hidroliza residuos de 4-O-ácido metil glucurónico (4-Me-GlcA) localizados en los extremos no reductores de cadenas de β-1,6 galactosil de las AGPs (Haque et al., 2005).

Se ha demostrado la acción sinergística y coordinada de varias enzimas en la degradación eficiente de polisacáridos como pectina y hemicelulosa (Conejo-Saucedo et al., 2011). En cuanto a AGs tipo II se refiere, se tuvo la primera evidencia de esta acción sinergística cuando se purificó la endo-β-(1,6)-D-galactanasa de *A. niger,* la cual no mostró actividad sobre AGPs nativos debido a que no habían sufrido una remoción preliminar de residuos de arabinosa (Brillouet et al., 1991). Por lo anterior, en los reportes posteriores donde se evaluó actividad enzimática, se utilizaron sustratos previamente tratados con una α-L-arabinofuranosidasa (Okemoto et al., 2003; Kotake et al., 2004; Sakamoto et al., 2007;). Ahora se sabe que las α-L-arabinofuranosidasas son necesarias para la degradación completa de varios polisacáridos acelerando las reacciones y mejorando la eficiencia catalítica de otras enzimas (Numan & Bhoshe, 2006). Esto último se demostró cuando la acción combinada de la α-L-arabinofuranosidasa y la endo-β-(1,6)-D-galactanasa de *N. crassa* incrementaron sustancialmente la proporción de residuos liberados (Takata et al., 2010).

Por otra parte, se ha reportado que la acción combinada de cuatro enzimas (exo-β-(1,3)-D-galactanasa, β-(1,6)-D-galactanasa, α-L-arabinofuranosidasa y α-L-arabinopiranosidasa) presentó mayor efectividad en la degradación de AGs tipo II, que la suma de las actividades de las

enzimas por separado (Okawa et al., 2013). Este sinergismo depende de la remoción de las ramificaciones β-(1,6)-galactosa por la β-(1,6)-galactanasa (Sakamoto & Ishimaru, 2013).

4. Importancia biotecnológica de galactanasas y arabinofuranosidasas

Las enzimas se han convertido en herramientas insustituibles para la obtención industrial de productos de índole muy diversa. Enzimas de distinta naturaleza y actividad son utilizadas por muchas industrias de alimentos, químicas y farmacéuticas (Flores-Herrera et al., 2004). Las galactanasas particularmente están involucradas en procesos como el bio-blanqueamiento de fibras. Las capas exteriores de la PC de las fibras de pulpa están enriquecidas con lignina y galactanos unidos químicamente; por lo tanto y debido a su actividad hidrolítica de galactanos, las galactanasas contribuyen al blanqueo (Luonteri et al., 2003; Sakamoto & Ishimaru, 2013).

Otro ámbito en el que intervienen las galactanasas es en la ingeniería de las pectinas. Las propiedades físicas de este polisacárido son afectadas por sus ramificaciones pues la abundancia de cadenas laterales disminuye su capacidad gelificante; por ello, la hidrólisis enzimática de estas ramificaciones aumenta considerablemente la calidad de las pectinas (Sakamoto & Ishimaru, 2013)

Las aplicaciones biotecnológicas de las α-L-arabinofuranosidasas son más diversas. Debido a su acción sinergística con enzimas lignocelulolíticas y hemicelulolíticas se han convertido en herramientas importantes para varios procesos agro-industriales. Éstos incluyen la producción de compuestos medicinales, el mejoramiento del sabor de los vinos, la calidad del pan, el blanqueamiento de la pulpa, la clarificación de jugos de fruta, asimilación del forraje, producción de bioetanol y síntesis de oligosacáridos (Numan & Bhoshe, 2006).

IV.HIPÓTESIS

Existen diferencias en la regulación y expresión de los genes de la α-L-arabinofuranosidasa y la endo-β-(1,6)-D-galactanasa, así como en la actividad enzimática asociadas con diferentes estilos de vida de dos razas de *C. lindemuthianum.*

V. OBJETIVOS

General

Caracterizar los genes de la α-L-arabinofuranosidasa y la endo-β-(1,6)-D-galactanasa, en relación con su expresión en dos razas de *Colletotrichum lindemuthianum* con diferentes estilos de vida.

Particulares

- Identificar y analizar los genes de la α–L-arabinofuranosidasa y endo-β-(1,6)-D-galactanasa de *C. lindemuthuianum*.

- Analizar la regulación y expresión de los genes de la α–L-arabinofuranosidasa y endo-β-(1,6)-D-galactanasa de *C. lindemuthuianum*.

- Evaluar la actividad enzimática de la α–L-arabinofuranosidasa en dos razas de *C. lindemuthianum*.

- Purificar y caracterizar la α–L-arabinofuranosidasa de *C. lincemuthianum*, raza 1472.

VI.RESULTADOS

Capítulo 1

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The role of virulence factors in the pathogenicity of *Colletotrichum* sp.

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Abstract: The *Colletotrichum* genus has been considered as one of the top 10 fungal pathogens in molecular plant pathology based on their scientific and agrobiological importance. Although the genus contains species with different lifestyles, most of the *Colletotrichum* sp. are known by their hemibiotrophic strategy of infection/invasion causing anthracnose disease in many economically important crops. Hemibiotrophy includes two sequential stages of infection, biotrophy and necrotrophy, in a series of steps that involve the participation of different virulence factors. In this review, we present the current status of the knowledge of such factors reported in this genus and a list of related genes identified in *Colletotrichum* sp. genomes.

Keywords: Biotrophy, *Colletotrichum* sp, hemibiotrophy, infection/invasion, necrotrophy, virulence factors.

1. INTRODUCTION

The species of *Colletotrichum* genus are included in the morphological classification of the *Ascomycota*. The genus comprises around a hundred species, although it is likely that further *Colletotrichum* taxa remain unidentified in major groups that have not yet been the subject of comprehensive multilocus phylogenetic studies [1]. *Colletotrichum* sp. present different lifestyles, such as of plant saprophytes [2, 3], plant pathogens with biotrophic, necrotrophic and hemibiotrophic invasion strategies, endophytes of conifers and ferns [1, 4-9], entomopathogenics [1, 10] and, exceptionally, as etiological agents of keratitis and subcutaneous infections in humans and a mycotic infection of a sea turtle [1, 11-15]. However, the genus is characterized mainly by plant pathogenic species, primarily described as causing anthracnose disease with a wide variety of host ranges and different levels of virulence from saprophytes to destructive pathogens that affect many economically important crops such as strawberries, mangoes, citrus, beans, avocados, bananas, coffee, maize, sugar cane and sorghum [1, 16-18]. Symptoms of anthracnose disease include limited and commonly submerged necrotic lesions on stems, leaves, flowers and fruits, besides the seedling blight, crown and stem rot [1, 19, 20].

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According to the well known models of plant pathogens such as the hemibiotrophic species *C. lindemuthianum* and *C. orbiculare*, the conidia of these fungi adhere and geminate on plant surfaces, produce germ tubes and form appressoria, which penetrate the host epidermal cell in susceptible cultivars. Appressoria subsequently develop an infection vesicle and extend into adjacent cells by means of large primary hyphae, which invaginate without penetrating the cell membrane and thus persisting as a biotrophic interaction. Once a large area of the plant tissue has been colonized, necrotrophic hyphae develop causing an extensive degradation of cell walls by the action of a variety of secreted enzymes and later the death of host cells [21-23].

In this review, we present the current status of the knowledge of virulence factors reported in *Colletotrichum* sp. for the stages of infection/invasion biotrophy and necrotrophy, such as pre-penetration and penetration of the host surface, effectors, proteins related to signal transduction pathways, transcription factors and cell wall-degrading enzymes. Additionally, we present a list of genes that encode the virulence factors identified in *Colletotrichum* sp. genomes.

2. PRE-PENETRATION PROCESSES OF THE HOST SURFACE

The pre-penetration includes the adhesion and germination of the fungus and the formation of an

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appressorium [24, 25]. In particular, the few studies of virulence factors that participate in pre-penetration have only been carried out on *C. gloeosporioides*, a pathogen of citrus, tomato, and avocado, among others. Thus, based on the transcripts expressed during the confrontation of the fungus with tomato and avocado fruits, the cDNA of the *cap20* gene was isolated. This gene encodes an appressorial cell wall protein whose genetic interruption causes a steep decrease in the virulence of the fungus [26]. Additionally, the isolation and characterization of three genes (*Cap3, Cap5* and *Cap22*), which are exclusively expressed in the conidia during the process of appressorium formation, have led to the identification of a copper metallothionein (Cap3), a metallothionein (Cap5) and a protein located in the appressorium wall (Cap22), which did not show homology with any other protein reported so far. Although these genes are considered as pathogenicity factors, the function of these proteins in fungal virulence was not established (Table 1) [27].

3. PENETRATION OF THE HOST SURFACE

The epidermal cells of aerial plant components are covered on their external surface by cutin, which is a polymer network of C_{16} and C_{18} oxygenated fatty acids bound by ester bonds. The role of this polymer is to protect the plant from water loss, irradiation, and xenobiotics and constitutes the first physical defense barrier against pathogens [28, 29].

In general, phytopathogenic fungi have developed two mechanisms to penetrate the host's cuticle: an enzymatic process and mechanical penetration. The first mechanism is performed using enzymes that degrade cutin, known as cutinases [24], which are considered as virulence factors of phytopathogenic fungi according to the Database of Virulence Factors in Fungal Pathogens (DFVF) (http://sysbio.unl.edu/DFVF/). Therefore, these enzymes have been studied in several phytopathogenic fungi, including *Colletotrichum* sp. (Table 1). In initial studies, it was observed that *C. gloeosporioides* penetrated a papaya's cuticle layer by secreting cutinase. When a papaya was treated with the cutinase purified from the fungus and subsequently inoculated with spores from the papaya pathogen *Mycosphaerella* sp., infection and lesion formation were favored [30]. Additionally, mutant strains of *C. gloeosporioides* without cutinase were unable to infect intact papaya surfaces. However, when causing a previous surface lesion or when the papaya was pre-treated with cutinase and inoculated with the mutant, typical anthracnose lesions were produced, thus confirming that the degradation of the papaya's cuticle by a cutinase is vital for fungal virulence [31]. It was subsequently established that *C. lagenarium* produces at least two types of cutinases when growing as a saprophyte using cutin as a substrate, thus producing at least one enzyme in the acervulus matrix during sporulation [32]. Moreover, *C. graminicola,* a maize pathogen, produces four cutinases located in the mucilage surrounding the acervuli

[33], and *C. gloeosporioides* secretes a cutinase in the infection peg derived from the appressorium [34]. However, microscopic observations of the penetration and colonization of *C. lagenarium* in cucumbers in the presence of cutinase inhibitors and mutants without the protein revealed that cutinases have no primary role in the penetration mechanism in this model. Additionally, little correlation between cutinase production and penetration or disease development was observed [35], thus suggesting that the enzymatic hydrolysis of cutin is not a general penetration mechanism in all *Colletotrichum* sp.

Table 1 shows the cutinases in which the gene has been characterized and the proteins have been purified [30, 36, 37], and Table 3 shows the cutinases that have been reported in databases. However, there is no exact knowledge of functionality. It was not possible to identify sequences similar to cutinases in all genomes of *Colletotrichum* available in NCBI (Table 2).

The second mechanism involved is the mechanical penetration of the cuticle because of the turgor pressure caused by the appressorium on the plant's cuticle [24]. In species such as *C. lagenarium* and *C. lindemuthianum* (a pathogen of *Phaseolus vulgaris*), penetration of the plant's cuticle and cell wall involves a combination of mechanical force produced by high turgor pressure and enzymatic degradation [25]. However, the mechanical and enzymatic combination is not widespread. It has been demonstrated that in *C. kahawae*, turgor pressure plays a more important role than cutinases in the penetration of the cuticle in coffee plants [38]. The appressorium turgor pressure is generated by the accumulation of solutes, mainly glycerol, from the metabolism of fatty acids in the spore's lipid coat. Glycerol is synthesized in large amounts in the appressorium and generates the mechanical pressure necessary to penetrate the plant's cuticle [39-41]. Although glycerol accumulation in the appressorium has been described in detail in the phytopathogenic fungus *Magnaporthe grisea*, it is proposed as a general process for other phytopathogenic fungi [42].

Another key point for the penetration of the host surface is the melanization of the appressorium. Such a pigment confers rigidity and the mechanical support to penetrate the plant's tissue [43]. Given the relevance of appressorium melanization, several studies have been performed on the biosynthesis pathway of this pigment, with *C. lagenarium* being the best-studied model. Using mutant strains of this fungus without melanin, generated by irradiation with ultraviolet light and chemical mutagens, it was demonstrated that the pigmentation of the appressorium walls is vital to form the penetration hyphae and therefore to successfully penetrate the cuticle [44-46]. Subsequently, inhibition of melanization of the appressorium of *C. lagenarium* with cerulenin revealed that the pigment's synthesis is performed through a polyketide biosynthesis pathway [47]. Genes encoding key proteins for the pathway and are vital for fungal pathogenicity were identified and thus considered as virulence factors (Table 1) [48].

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Table 1 Virulence factors of *Colletotrichum* sp.

Step	Virulence factor	Gene	Specie	Protein Access number	Reference
Pre-penetration of host surface	Appressorial cell wall protein	*Cap20*	*C. gloeosporioides*	AAA77678.1	[26]
			C. gloeosporioides	AFR58872.1	Unpublished
			C. acutatum	AFR58869.1	Unpublished
	Appressorial cell wall protein	*Cap22*	*C. gloeosporioides*	AAA77681.1	[27]
	Copper metallothionein	*Cap3*	*C. gloeosporioides*	AAA77679.1	[27]
	Metalloprotein	*Cap5*	*C. gloeosporioides*	AAA77680.1	[27]
Penetration of host surface	Cutinase	*CutA*	*C. capsici*	AAA33043.1	[36]
			C. truncatum	ADQ27862.1	Unpublished
			C. gloeosporioides	AAL38030.1	[30, 37]
	Polyketide synthase	*Pks1*	*C. lagenarium*	BAA18956.1	[49]
	Scytalone dehydratase	*Scd1*	*C. lagenarium*	BAA13009.1	[51]
	Tetrahydroxynaphthalene reductase	*Thr1*	*C. lagenarium*	BAA18962.1	[52]
	Laccase	*Lac2*	*C. orbiculare*	BAN13563.1	[53]
	Tetraspanin	*Pls1*	*C. lindemuthianum*	CAD43407.1	[54]
	Autophagy-related protein 26	*Atg26*	*C: lagenarium*	BAH60889.1	[55]
Signal transduction	Putative serine/threonine kinase	*Clk1*	*C. lindemuthianum*	AAB61403.1	[77]
	MAP kinase	*Cmk1*	*C. lagenaria*	AAD50496.1	[78]
			C. gloeosporioides	BAB21569.1	Unpublished
	MAP kinase kinase	*CgMEK*	*C. gloeosporioides*	AAD55385.1	[79]
	cAMPdependent protein kinase catalytic subunit	*Ct-PKAC*	*C. trifolli*	AAC04355.1	[80]
			C. lagenaria	BAD04044.1	Unpublished
			C. gloeosporioides	ABG89386.1	[81]
Transcriptional factors	Transcriptional activator 1	*CLTA1*	*C. lindemuthianum*	AAG25917.1	[82]
	Ste12	*CST1*	*C. lagenarium*	BAC1180	[84]
			C. lindemuthianum	CAD30840.23.1	Unpublished
	Ste12-like	*CLSTE12*	*C. lindemuthianum*	CAL38822.1	[85]
	pH-responsive PacC	*PacC*	*C. acutatum*	ABL96218.1	[86]
			C. lindemuthianum	AFU83185.1	Unpublished
Hydrolytic enzymes	Pectate lyase		*C. magna*		[113]
			C. gloeosporioides		[124]
		Pel	*C. gloeosporioides*	AF052632	[89]
			C. gloeosporioides		[125]
		PelB	*C. gloeosporioides*	AF052632	[127]
		PecCl1	*C. lindemuthianum*		[126]
		CcpelA	*C. coccodes*		[128]
	Pectin lyase	*PnlA*	*G. cingulata*	AAA21817	[92]

The polyketide synthase protein encoded by gene *pks1*, which catalyses the first step in the pathway by synthetizing polyketide (1,3,6,8-tetrahydroxynaphthalene) from acetyl CoA or malonyl CoA, was identified [43, 49]. Additionally, the 1,3,6,8-tetrahydroxynaphthalene reductase that reduces polyketide in *C. lagenarium* [50] was identified with gene *scd1*, which encodes a scytalone dehydratase that converts intermediary scytalone into 1,3,8-THN [51], and gene *thr1*, which encodes tetrahydroxynaphthalene reductase to convert 1,3,8-THN to vermelone [52]. *C. lagenarium* mutants without any of these proteins showed an albino genotype with little or no infection capacity. Table 2 shows a list of genes encoding the proteins involved in the pathway of melanin biosynthesis, as identified in the genomes of *Colletotrichum* sp. available in the NCBI database. Additionally, the inhibition of melanization decreases the turgor pressure of the appressorium and consequently the fungal virulence in *C. kahawae* [38].

Other genes for proteins involved in virulence, associated with melanization or appressorium formation, include a laccase, ATPase and tetraspanin. *C. orbiculare* (a cucurbitaceae pathogen) mutants in gene *lac2* encoding the laccase Lac2 that oxidizes 1,8-DHN, lose pathogenicity because the appressorium is not functional [53].

Table 2. Genes of virulence factors identified in genomes of *Colletotrichum* sp.

Virulence factor	Gene	*Colletotrichum orbiculare* MAFF 240422	*Colletotrichum higginsianum*	*Colletotrichum sublineola*	*Colletotrichum gloeosporioides* Cg-14	*Colletotrichum fioriniae* PJ7	*Colletotrichum graminicola* M1.001	*Colletotrichum gloeosporioides* Nara gc5
Appressorial cell wall protein	*Cap22*	ENH77144.1	CCF46233.1	KDN62809.1	EQB51872.1	EXF80893.1	EFQ31127.1	ELA38273.1
Copper metallothionein	*Cap3*				EQB53345.1	EXF74198.1		
Appressorial cell wall protein	*Cap20*	ENH83379.1	CCF45503.1	KDN69556.1	EQB50426.1	EXF84715.1	EFQ33405.1	ELA28168.1
Cutinase	*CutA*	ENH76872.1				EXF73863.1		ELA29687.1
Polyketide synthase	*Pks1*	ENH81867.1	CCF45141.1	KDN65285.1	EQB55056.1	EXF80059.1	EFQ29059.1	ELA23922.1
Scytalone dehydratase	*Scd1*	ENH88853.1	CCF36486.1	KDN67717.1	EQB50834.1	EFQ32411.1	EFQ32411.1	
Tetrahydroxynaphthalene reductase	*Thr1*	ENH87277.1	CCF34762.1	KDN64838.1|	EQB59200.1	EXF85981.1	EFQ32857.1	ELA35640.1
Laccase	*Lac2*	ENH79795.1	CCF41971.1	KDN63814.1	EQB47848.1	EXF76206.1	EFQ31449.1	ELA27891.1
Autophagy-related protein 26	*Atg26*		CCF46551.1	KDN68897.1	EQB44290.1	EXF77084.1	EFQ27252.1	ELA29771.1
Tetraspanin	*Pls1*	ENH86991.1	CCF41127.1	KDN61862.1	EQB43541.1	EXF85044.1	EFQ26732.1	ELA35212.1
Transcriptional activator 1	*CLTA1*	ENH84236.1	CCF41784.1	KDN65607.1	EQB49973.1	EXF78226.1	EFQ35927.1	ELA27031.1
Ste12	*CST1*	ENH85200.1	CCF46069.1 BAJ23071.1	KDN65393.1	EQB56368.1	EXF77490.1	EFQ27157.1	ELA26856.1
Ste12-like	*CLSTE12*	ENH79208.1		KDN67993.1	EQB51947.1	EXF73496.1	EFQ33119.1	ELA24448.1
pH-responsive PacC	*PacC*	ENH85250.1		KDN64520.1	EQB54743.1	EXF80715.1	EFQ28428.1	
cAMPdependent protein kinase catalytic subunit	*Ct-PKAC*	ENH78338.1	CCF47880.1	KDN72000.1	EQB45795.1		EFQ28594.1	ELA34572.1
Putative serine/threonine kinase	*Clk1*	ENH78609.1	CCF37471.1	KDN63867.1	EQB49072.1	EXF84063.1	EFQ25054.1	ELA34992.1
MAP kinase	*Cmk1*		CCF37063.1		EQB44162.1	EXF85614.1	EFQ31798.1	ELA38156.1
MAP kinase kinase	*CgMEK*	ENH81835.1	CCF40893.1	KDN61133.1	EQB46387.1	EXF78594.1	EFQ29115.1	ELA30304.1
Polygalacturonases			CACQ02000936	JMSE01000799	XM_007286512 XM_007276494 XM_007275337	XM_007599435, XM_007593210, XM_007597050	GG697355	
Pectate lyase			CACQ02008115	JMSE01000302	XM_007282661, XM_007281377, XM_007279259	XM_007589695, XM_007594351, NW_006888742	2063512275	
Pectin lyase			CACQ02001292	JMSE01001002, JMSE01001319	XM_007279216, XM_007273511	XM_007601225, XM_007591291, NW_006889667	GG697353, GG697353	
Pectin methylesterase			CACQ02002831, CACQ02007217, CACQ02003036	JMSE01000056	XM_007287240	XM_007596854, XM_007590868, XM_007601775	GG697397, GG697343	
Xylanase	GH11	ENH76821.1 ENH79971.1 ENH89093.1	CCF32229.1 CCF46991.1	KDN62561.1 KDN71533.1 KDN62296.1 KDN69962.1	EQB58440.1 EQB44117.1	XP_007597263.1 XP_007592531.1 XP_007592808.1	EFQ27362.1 EFQ27481.1 EFQ30380.1 EFQ27243.1	XP_007277672.1 XP_007273422.1
	GH10	ENH76371.1	CCF41074.1	KDN69189.1	EQB54800.1	XP_007590417.1	EFQ33770.1	XP_007285158.1
Serine threonine protein kinase		ENH81932.1	CCF38312.1	KDN61158.1	EQB56372.1	XP_007597786.1	EFQ29088.1	XP_007285050.1

Using genome sequence analyses of *C. orbiculare, C. graminicola* and *C. higginsianum* available in databases, one or two genes homologous to *lac2* were identified for each species, which suggests that the function of this laccase may be conserved in the *Colletotrichum* genus (Table 2). The DFVF considers tetraspanin a virulence factor for *Colletotrichum*. The transcripts of this protein are located specifically in the conidia, germinal tube and appressorium of the necrotrophic fungi *Botrytis cinerea* and are vital for appressorium penetration as mutants lacking this gene were incapable of penetrating intact plant surfaces [54]. Although it is proposed that tetraspanin carry out the same function in many fungal species and was identified in *Colletotrichum* genomes (Table 2), it is necessary to perform studies to confirm its role in virulence.

Additionally, the mutation in gene *Atg26* in *C. orbiculare*, which encodes a sterol glucosyltransferase, an autophagy-related protein required for ergosterol glucoside biosynthesis involved in pexophagy (Table 1), shows normal appressorium development. Nevertheless, the fungus is incapable of invading the host's tissue and peroxisome autophagy greatly decreases in the structure.

These results suggest that *Atg26* is involved in peroxisome homeostasis in the appressorium, indicating the physiological importance of pexophagy in host invasion by the fungus. Therefore, it was proposed that *Atg26* is a factor required for the pathogenicity of *C. orbiculare* and in the development of anthracnose disease symptoms in cucumber [55].

4. EFFECTORS AND EVASION OF THE PLANT IMMUNE SYSTEM

Plants respond to an infection using the innate immune system through two pathways. The first pathway recognizes pathogen associated molecular patterns (PAMPs and MAMPs) using pattern recognition transmembrane receptor (PRRs) and is known as PAMP-triggered immunity (PTI). The second pathway responds to pathogen-specific virulence factors known as effectors, which are secreted into the cytoplasm and can be recognized by polymorphic NB-LRR proteins (R proteins). This process is known as effector-triggered immunity (ETI) [56].

Table 3. Cutinases reported in NCBI for the *Colletotrichum* genus.

Colletotrichum fioriniae PJ7	*Colletotrichum gloeosporioides* Nara gc5	*Colletotrichum gloeosporioides* Cg-14	*Colletotrichum gloeosporioides*	*Colletotrichum graminicola* M1.001	*Colletotrichum higginsianum*	*Colletotrichum orbiculare* MAFF 240422	*Colletotrichum truncatum*	*Colletotrichum capsici*
EXF84454.1	ELA30029.1	EQB59021.1	AAA33042.1	EFQ36028.1	CCF32012.1	ENH87041.1	ADQ27862.1	AAA33043.1
EXF84429.1	ELA29687.1	EQB58673.1	1402192B	EFQ35915.1	CCF33699.1	ENH80803.1		1402192A
EXF83572.1	ELA26721.1	EQB58498.1	AAL38030.1	EFQ35324.1	CCF35717.1	ENH76872.1		
EXF82041.1	ELA26696.1	EQB55558.1		EFQ34785.1	CCF37658.1			
EXF78254.1	ELA25638.1	EQB54919.1		EFQ34434.1	CCF38096.1			
EXF78236.1		EQB53657.1		EFQ34152.1	CCF39674.1			
EXF77964.1		EQB50526.1		EFQ33196.1	CCF40758.1			
EXF75966.1		EQB49110.1		EFQ31522.1	CCF41430.1			
EXF75869.1		EQB47776.1		EFQ30654.1	CCF41797.1			
EXF75131.1		EQB46221.1		EFQ26957.1	CCF43068.1			
EXF74344.1		EQB45230.1		EFQ26949.1				
EXF73874.1		EQB44664.1		EFQ25748.1				
EXF73863.1		EQB44224.1						
EXF72939.1		EQB44152.1						

The function of the effectors is closely related to the strategy of nutrition/infection of the pathogen. For example, the main functions of biotrophic fungi effectors are in the suppression or evasion of the second line of immune system response (ETI) and the manipulation of the host's metabolism to increase nutrient availability for the pathogen, thus guaranteeing its permanence and survival. However, the effectors of necrotrophic fungi include specific toxins that promote rapid cell death [57, 58]. Therefore, one could suppose that hemibiotrophic fungi (most *Colletotrichum* sp.) secrete different effectors during the bio- and necrotrophic phases. In this regard, Kleeman et al., (2012) performed a notable study regarding the expression of genes encoding effector candidates during the interaction of *C. higginsianum* with *Arabidopsis thaliana* in the different stages of infection development. The analysis of the expression of 17 genes by qRT-PCR indicated that four successive expression peaks of effector genes occur. The first peak is observed in the appressorium, which has not penetrated the plant. Other effectors begin to express in this stage but their expression is constant in the biotrophic phase (second peak). Other effectors are specifically induced during the penetration of the plant's surface and during differentiation into primary hyphae (third peak). The final expression peak corresponds to effectors that are putative toxins and occurs during the change to necrotrophy, thus suggesting that the toxic products generated by the action of these effectors contributes to the end of the biotrophic phase and the start of the necrotrophic phase. Notably, there are other effectors that are exclusively induced in the mycelia of the saprophyte *C. higginsianum*, which could indicate that effectors are differentially expressed depending on the lifestyle of the fungus (pathogen or saprophyte) [59, 60]. Changes in the expression pattern of genes that are effector candidates regarding the stage of infection development (pre-penetration, penetration, biotrophy and necrotrophy) have also been reported for other *Colletotrichum* sp. *(C. graminicola, C. higginsianum, C. orbiculare* and *C. gloeosporioides)* [61, 62].

Similarly, the effector CtNUDIX was identified (*Colletotrichum truncatum* NUcleoside DIphosphate linked to some other moiety X). The gene of this effector (*CtNUDIX*) is exclusively expressed during the biotrophic phase precisely before the change to the necrotrophic phase (a critical step in the development of the infection) and can promote cell death similarly to a hypersensitive response in tobacco leaves with temporal expression of the gene. However, the inactivation of any domains of the protein causes a loss of function, and the overexpression of the gene causes an incompatible interaction with the host because the accumulation of this effector causes increased cell death [63]. Additionally, a predicted peptide was identified in the secretome of *C. truncatum* with a NUDIX hydrolase domain. This result suggests that homologues of this effector (containing a signal peptide and NUDIX hydrolase domain) could be exclusive to hemibiotrophic fungi because promoting cell death as a signal for the transition of biotrophy to necrotrophy has been attributed to them [63, 64].

ClH1 (*Colletotrichum* Intracellular Hypha 1) is a proline-rich glycoprotein that is structurally similar to proline- and hydroxyproline-rich proteins (PRPs and HRGPs), which are typical proteins in the plant cell wall. Gene *ClH1* isolated

from *C. lindemuthianum* and subsequently identified in *C. higginsianum* are exclusively expressed during the biotrophic phase of the infection, and the protein has been located in the interfacial matrix of the primary hyphae but not in the spores or appressorium. This result suggests that fungi modify their surface composition most likely to prevent pathogen recognition of the host's cells because of the separation of the plasmatic membrane of the plant and fungi's cell wall. Another possibility is that because of their structural similarity to PRPs and HRGPs in the plant, they could mimic the composition of the plant cell wall to avoid being recognized by the host as a pathogen. Finally, ClH1 could also act as a physical barrier against the host's defense molecules [59, 65, 66]. A lysin motif (LysM) was identified in protein ClH1, a domain whose function is to recognize N-acetylglucosamine (GlcNAc) [67]. LysM is typical of effectors and is highly conserved in several fungal species. The function of effectors with LysM domains is the sequestration of chitin oligosaccharides (products of the fungal cell wall degradation), which are released during the invasion and activate the plant's defense responses against the pathogen [68].

Another important report regarding *Colletotrichum* effectors corresponds to the isolation of gene *CgDN3* from *C. gloeosporioides*. This gene showed homology with an internal region of plant wall-associated receptor kinases. Mutants showed a fast mycelial growth *in vitro* and the production of viable spores and formation of the appressorium in the leaf's surface. However, mutants were incapable of infecting and reproducing and caused a reaction similar to a hypersensitive response located in the intact leaves of the host *Stylosanthes guianensis*, suggesting that *CgDN3* encodes a possible effector associated with the biotrophic phase that is required to evade the defense responses of a compatible host [69]. In *C. higginsianum,* effectors are secreted from the penetration pores of the appressorium in a highly polarized manner before the host invasion, and the primary hyphae have an important role in the secretion of effectors to counteract the plant's defenses (*C. higginsianum* can suppress callose deposition) and prepare colonization of the host cell [59].

The analysis and sequencing of genomes and transcriptomes of some *Colletotrichum* sp. has provided important information in several aspects. In these studies, it has been possible to identify effector candidates, characterized as extracellular soluble proteins of < 300 aa and rich in cysteine [60]. According to these criteria, 11 secreted proteins were identified in the transcriptome of *C. truncatum* and catalogued as effectors [64]. Two proteins with LysM domains (contig 5, Accession no. ACF194227 and contig Ct21-1573) were identified as part of these effector candidates. The first protein was similar to the LysM effector protein (ECP6) of *Cladosporium fulvum*; these effectors may play an important role in the evasion of the host's defense response as previously mentioned [70]. The second protein (contig 8, Accession No. ABE73692) corresponded to a homologue of an eliciting plant response-like protein from *Ceratocystis fimbriata*, which contains a CP (cerato-platanin) domain. Although the function of this effector candidate was not established, it could act as a signal molecule in the activation of defense response, such as the synthesis of phytoalexins in *C. fimbriata* [71].

The analysis of candidate secreted effectors (CSEPs) from the transcriptomes of *C. graminicola* and *C. higginsianum* indicated 177 CSEP-encoding genes, of which 85 (48%) were species-specific. Furthermore, in *C. higginsianum*, twice this amount of CSEPs (365) were identified, including a higher number (264, 72%) of effector candidates specific to species. The difference in the number of CSEP identified in each species was explained as an adaptation to a wide range of hosts of *C. higginsianum* compared to *C. graminicola*, which is restricted to one host under specific culture conditions [61]. A comparative genomic and transcriptomic study performed in *C. graminicola, C. higginsianum, C. orbiculare* and *C. gloeosporioides* determined that *C. orbiculare* and *C. gloeosporioides* possess 372 and 355 cysteine-rich (< 3%) secreted proteins, respectively. Of these, 318 and 288 are < 300 aa, which classifies them as effector candidates. These results are consistent with the high proportion of effector candidates of *C. graminicola* and *C. higginsianum* [62].

Among the transcriptomes analyzed from the previously mentioned *Colletotrichum* sp., specific effector candidates were located and showed similarities with other previously reported effectors. Such is the case for effector candidate ChEC5 of *C. higginsianum* that showed 79% similarity with effector MSP1 (*Magnaporthe grisea* snodprot1 homolog 1) of *M. grisea* [59]. This protein was considered an important virulence factor because mutants in this gene showed an important decrease in virulence. However, the protein does not possess apparent phytotoxicity or protease activity; therefore, the function of this effector is unknown [72]. Another *C. higginsianum* effector candidate is ChEC88, which has 49% similarity with the BAS (Biotrophy Associated Secreted) protein 3 of *Magnaporthe oryzae* [59]. Using these results, it was possible to identify the BAS protein 2 effector in the analyzed secretomes of *C. orbiculare* and *C. gloeosporioides* [62]. The BAS effectors are proteins that accumulate in the interfacial complexes of the biotrophic hyphae of *M. oryzae* [73]. The SIX (Secreted in Xylem 5 protein) effectors were identified in most transcriptomes of *Colletotrichum*. SIX 1, 5 and 6 (ChEC 36 of *C. higginsianum*) showed 40% similarity with the *F. oxysporum* sequence [59, 62]. These effectors promote the virulence of *F. oxysporum* in tomato plants, and their combined presence and absence most likely determines host specificity and the activation of defense responses [74]. Finally, in *Colletotrichum* transcriptomes, effector candidates with NPP1 (Necrosis-inducing proteins) domains were identified, which have been termed NLP (Nep1-like protein) proteins and are associated with the necrotrophic phase of the infection (ChNLP1 and ChNLP3 of *C. higginsianum*) [59, 62]. Although the function of these proteins has not been specified, evidence suggests that they are virulence factors that accelerate the disease and favor pathogen growth in the host [75].

Although important information regarding effectors has originated from genome and transcriptome sequencing, it is necessary to determine their function during plant-pathogen interactions, which could help to design new control strategies for diseases caused by *Colletotrichum* sp.

5. SIGNAL TRANSDUCTION

Among the signaling pathways involved in *Colletotrichum* sp. virulence is the Ca^{2+}/calmodulin signal transduction pathway. Altering the homeostasis of calcium or the chemical blocking of calcium channels affects the development of the appressorium in *C. trifolii* and calmodulin inhibitors affect both the germination and spore differentiation of the pathogen. Therefore, the transduction pathway is potentially involved in the pre-penetration process of the host's surface [76].

Moreover, the gene *clk1* (*Colletotrichum lindemuthianum* kinase 1) was identified in *C. lindemuthianum* using random mutagenesis assays. This gene encodes a protein belonging to the serine/threonine protein kinase family (Table 1), whose function is fundamental in the initial steps of the infection process [77].

Regarding the MAP kinase pathway (MAPK), a MAPK encoded by gene *Cmk1* was identified and isolated from *C. lagenarium*. The mutation of this gene resulted in the complete loss of virulence of the pathogen because it was incapable of germinating in both the plant's surface and artificial surfaces. Additional analyses suggest that *Cmk1* regulates several pathogenesis stages, including the formation of the appressorium. The gene *Cmk1* is conserved in other fungal species, such as *M. grisea*, which suggests that the function of this protein is important in the formation of the appressorium, thus forming a generalized signaling pathway involved in the formation of fungal infection structures [78]. Additionally, the mitogen-activated protein kinase (MEK), CgMEK of *C. gloeosporioides* (Table 1), was evaluated. The interruption of gene *CgMEK* resulted in the loss of its ability to form an appressorium and therefore the loss of virulence. Confocal microscopy determined that MEK is involved in two key processes during the differentiation of the appressorium, the first of which corresponds to polarization during cell division and septa formation and the second in the differentiation of the germinal tube. CgMEK1 is required for the differentiation of infection structures [79].

Another important protein component of the signaling pathways that has been studied is protein kinase A (PKA) (Table 1). Disruption by replacement insertion of the gene encoding the catalytic subunit C (*Ct-PKAC*) in *C. trifolii* showed a small reduction in the growth of the transformed strains compared to the wild type fungus; conidiation patterns were altered and the pathogen was incapable of infecting intact surfaces of alfalfa plants. PKA has an important role in the regulation of the transition between the vegetative growth and conidiation in *C. trifolii*; therefore, it is essential in the development of pathogenicity [80]. A similar study carried out with *C. gloeosporioides* concluded that although the mutants of this gene could form an appressorium, formation was delayed compared to the wild type. Additionally, the conidia of the mutant showed bipolar germination after the formation of the appressorium, but no appressorium was formed from the second germinal tube. It was possible to observe a reduced ability to adhere to a hydrophobic surface and degrade the lipids located in the appressorium and therefore a reduced ability to infect mango plants. This result confirmed that this protein plays an important role in the regulation of morphogenesis and is required for pathogenicity [81]. The proteins mentioned previously were identified in the genomes of genus *Colletotrichum* and are listed in Table 2.

6. TRANSCRIPTION FACTORS

Among the virulence factors of the *Colletotrichum* sp. reported in the DFVF, some are related to the regulation of genes encoding hydrolytic enzymes. Gene *CLTA1* (for *C. lindemuthianum* transcriptional activator 1), which encodes for a GAL4-like transcriptional activator, appears to be involved in the differentiation of primary hyphae into secondary hyphae during the interaction of *C. lindemuthianum* (H433) with *P. vulgaris* (Table 1). Therefore, it is considered that *CLTA1* is involved in the transition from biotrophy to necrotrophy, which is a key moment in the fungal infection, and it has been catalogued as a regulatory and pathogenic gene in *C. lindemuthianum* [82].

Ste12 and Ste12-like are transcription factors typical of fungi involved in the regulation of development and pathogenicity [83]. The genes encoding these two proteins were isolated and characterized in *Colletotrichum* sp. (Table 1). In the first case, the *CST1* gene encoding a homologous gene *Ste12* was isolated from *C. lagenarium*. The analysis of *cst1* knockout mutants demonstrated that CST1 plays an important role in the penetration of the appressorium [84]. Moreover, the *CLSTE12* (*Ste12*-like) gene of *C. lindemuthianum* corresponds to a transcription factor that is also essential for appressorium penetration. Proximal elements recognized by Ste12 in the promoter of the endopolygalacturonase gene were identified, suggesting that it is involved in gene expression during culture growth with pectin as the only carbon source and during pathogenesis [85].

It was reported that PacC is involved in the physiological regulation and development of infection structures of *C. acutatum* (Table 1) [86], and afterwards, it was possible to identify this transcription factor in *C. gloeosporioides*. It is estimated that approximately 5% of the pathogen genome is regulated by PacC including transporters, antioxidants and enzymes that degrade the cell wall. PacC is a key regulator of pathogenicity genes during the pH change in the infection process. Thus, under alkaline pH conditions, PacC acts as a positive regulator promoting the transcription of genes expressed under alkaline conditions, and a simultaneous repression of genes expressed under acid conditions is also suggested. Among those genes whose regulation depends on PacC, there are some encoding enzymes that degrade the cell wall (pectinases specifically), which are considered virulence

factors. Therefore, the function of this transcription factor is determinant in the fungal virulence and during the biomass conversion process of the host [87]. These transcription factors were located in the genomes of *Colletotrichum* sp. (Table 2).

7. CELL WALL DEGRADING ENZYMES

7.1. Pectate and pectin liases

Cell wall degrading enzymes (CWDEs) have been considered to play an important role in the process of plant pathogenesis of bacteria and fungi [88, 89]. The evidence for its role in pathogenesis includes the correlation between enzyme levels and virulence, immunocytochemical evidence for the secretion of the enzyme during infection, protection of the host by inhibition of the enzymes with specific inhibitors or specific antibodies and enhancement of virulence by gene transfer [90-95]. Particularly, pectinolytic enzymes are considered important in the pathogenesis because they are typically secreted early during this process, are able to macerate plant tissues killing plant cells by themselves [88, 96] and also because ultrastructural, biochemical and genetic studies have involved these enzymes in the processes of penetration, tissue maceration, nutrient acquisition, expression of characteristic symptoms of the disease and induction of plant defense responses [97-99]. Therefore, pectinases have been studied in more detail and in more pathogens than other depolymerases.

Pathogenic fungi produce a large amount of pectinase during pathogenesis, including polygalacturonases, pectin and pectate lyases and pectin methylesterases [100, 101]. The first evidence of the role of pectinolytic enzymes as virulence factors is based on the ability of these enzymes to produce disease symptoms when purified or partially purified enzymes are used [102-106] and the correlation found between the levels of pectinolytic enzymes with the extent of damage to the host and relative virulence [107-109].

A significant amount of work has been published on the role of pectolytic enzymes of fungi as virulence factors. The strategies that have been used are: I) inhibition by antibodies, II) UV or chemical mutagenesis, III) targeted mutagenesis, IV) gene complementation of a deficient mutant, V) heterologous gene expression, and VI) antisense gene expression. However, the results are highly variable and only a few studies have demonstrated their clear involvement in fungal pathogenicity [95, 110, 111].

Several studies that have used pectinase-deficient mutants obtained with UV light or chemicals have generated very contrasting results to those in which the contribution of pectinolytic enzymes with the disease symptoms was reported [112, 113]. Some studies using pectinase-deficient mutants led to the conclusion that these enzymes do not contribute in the development of disease symptoms [114-118]. Yet, the use of mutants generated in this manner has been questioned because it cannot be excluded that they carry additional mutations that somehow compensate loss of pectinases, or otherwise having additional pectinases [98].

Directed mutagenesis has been considered as the most direct way to investigate the role of pectinolytic enzymes as virulence factors. However, the effects vary widely. Targeted mutational studies of genes encoding polygalacturonase, pectate lyase or pectin methylesterases on *Cochliobolus carbonum* [90], *Cryphonectria parasitica* [119] and *Fusarium oxysporum* [120] showed no significant effect on virulence even when more than one of the genes encoding these enzymes were inactivated as in *C. carbonum* [94] and *B. cinerea* [121]. Others studies carried out in *Aspergillus flavus* [110], *B. cinerea* [111, 121, 122], *Alternaria citri* [97], *Claviceps purpurea* [123] and *Nectria hematococca* [95], reported a decrease in virulence of the mutant lines, and a decreased ability of plant tissue maceration.

Most of the investigations in the genus *Colletotrichum* have focused on the importance of pectate lyases as virulence factors than in the rest of the pectinolytic enzymes (Table 1). Wattad *et al.* (1994) used antibodies to inhibit the activity of pectate lyase during infection of *C. gloeosporioides* in avocado wedges mesocarp tissue both *in vitro* and *in vivo* obtaining a 80% of reduction of the activity of maceration [124]. The pectate lyase antibodies had no effect on spore germination, germ tube elongation or appressoria formation of *C. gloeosporioides*. However, when conidia were mixed with the antibodies prior to inoculation, symptom development was inhibited on avocado, mango and banana fruits [125]. Inhibition of avocado maceration capacity and inhibition of the development of symptoms suggest the importance of this enzyme in *C. gloeosporioides.*

The *pecCl1* gene encoding a pectate lyase of *C. lindemuthianum* was inactivated by the split-maker technique. Though gene inactivation did not result in complete loss of pectate lyase activity, the symptoms of anthracnose were reduced [126]. Disruption of the *pelB* gene encoding a pectate lyase of *C. gloeosporioides* by homologous recombination caused a reduction of 36 to 45% in estimated decay diameter when three mutant lines were inoculated onto avocado fruits in comparison with wild-type isolate and undisrupted transformed. The mutants did not produce or secrete pectate lyase B, pectate lyase activities and pectin lyase decreased 25%, while polygalacturonase activity increased 15% as compared to wild-type. However the ability to grow in glucose, sodium polypectate or pectin as a source of carbon was not reduced, except for 15% with pectin as the carbon substrate at pH 6 [127]. The fungi produce several enzymes capable of degrading pectin so a single gene disruption of the degradation complex is commonly offset by the activity of other enzymes [89, 90, 94, 119]. However, disruption of *pelB* caused a reduction in estimated decay diameter of infection in avocado fruits and the mutants induced a significantly higher host phenylalanine ammonia lyase activity, which is indicative of higher host resistance suggesting that PLB is an important factor in the attack of *C. gloeosporioides* on avocado fruit, and that its activity promotes the induction of host defense mechanisms [127].

C. magna is a pathogen of cucurbits able to cause only minor symptoms in watermelon seedlings and avocado fruits. To assess the contribution of pectate lyase in pathogenesis, the *pel* gene encoding a pectate lyase of *C. gloeosporioides* was expressed in *C. magna*. The transformants showed higher maceration capacity on avocado pericarp and even more in watermelon seedlings compared with the wild-type [89]. The disruption of *CcpelA* gene encoding a pectate lyase of *C. coccodes* isolates with different aggressiveness generated mutants that showed reduced aggressiveness towards tomato fruits and impaired PL secretion and extracellular activity whereas overexpression of a moderately aggressive isolate increased aggressiveness and pectate lyase secretion [128]. Wattad et al. (1995) reported a mutant of *C. magna* generated by UV unable to infect avocado. The mutant was unable to secrete pectate lyase, which was accumulated in the hyphae and generated low levels of this enzyme in the extracellular medium, suggesting that malfunctioning in the secretion of pectate lyase is related to the inability of infection [113]. Other authors have reported cases of deficiencies in the process of infection caused by alterations in the secretion of pectinolytic enzymes such as a *C. graminicola* mutant, which was obtained by random mutagenesis. The mutant, carrying a mutated *cpr1* gene encoding one component of the microsomal signal peptidase enzyme, failed to cause symptoms on either leaves or stalks of maize. It was however able to penetrate and colonize host cells during the biotrophic phase of the disease, but seemed unable to move to the necrotrophic phase. Because the decrease in transcript levels compared to wild type, it has been suggested that the mutant is unable to secrete degradative enzymes sufficient to switch phase [129]. The change to necrotrophic phase is associated with the development of secondary hyphae and the secretion of hydrolytic enzymes, such as pectinases, for macerating the walls of host cells [64]. These results suggest that pectate lyase is an important factor for maceration and is probably required during the early stages of pathogenesis in *Colletotrichum sp.* [89, 125].

Several studies have shown the importance of pH in pathogenicity. In concordance to the work of Yakoby and colleagues (2000), the host pH regulates the secretion of PL and may affect *C. gloeosporioides* pathogenicity [130]. The *pacC1* gene encoding the transcription factor PacC was characterized in *C. lindemuthianum* and observed that its transcription was activated by an elevated extracellular pH. The mutant line in *pacC* gene was able to penetrate the host tissue through differentiation of primary hyphae. However, it was not able to cause maceration on the infected plant tissue. These results suggest that PacCl is a regulator of gene activation, and its expression is required for fungal growth in alkaline conditions, as well as for the transcription of genes necessary for the passage from the biotrophic to the necrotrophic phase [131]. According to the above, the pH may be operating as an important regulator of the secretion of pectinolytic enzymes during infection.

With regard to the role of pectin lyases as virulence factors, the disruption the *pnlA* gene encoding a pectin lyase in *C. gloeosporioides* did not affect the ability to infect capsicum and apple, so it was considered that pectin lyase is not essential for pathogenesis [92]. However, this is the only report published on pectin lyases so far.

Another factor that has contributed to proposal that pectinases are virulence factors is the molecular dialogue between host and pathogen through the release of products by the action of pectinases, which are capable of inducing the defense system of plants and the presence of inhibitors of specific enzymes in plants [132]. The first polygalacturonase inhibitor protein (PGIP) was purified from bean hypocotyl extracts in 1971 by Albersheim and Anderson. The PGIP was able to inhibit the activity of a polygalacturonase of *C. lindemuthianum* more effectively than *F. oxysporum* and no inhibition was shown in *Sclerotium rolfsii* [133]. In *P. vulgaris*, four PGIPs (PvPGIPI1 to PvPGIPI4) with different recognition specificities against polygalacturonase from *B. cinerea*, *Aspergillus niger* and *Fusarium moniliforme* have been identified [134]. The presence of PGIP isoforms found in plants is thought to be an important factor for adaptation of plants to pathogens [134]. It has also been described a pectin lyase inhibitor protein (PNLIP) in sugar beet, which uncompetitively inhibits PNLs from the fungi *Rhizoctonia solani*, *Phoma betae* and *Aspergillus japonicus* [135]. Although pectate lyases have been mostly studied for their possible role as a virulence factors, there are no reported inhibitors for these enzymes. Yet, the flavonoid epicatechin that is present in high concentrations in the pericarp of unripe fruit was found to inhibit pectate lyase in *C. gloeosporioides* and so it is believed that it may regulate the activity of endopolygalacturonases produced during the infection process [124, 136]. Though the role of pectinolytic enzymes as virulence factors remains to be clarified, these enzymes and particularly pectate lyases are important during infection and in the case of hemibiotrophic fungi, they appear to be fundamental in the switch between biotrophic and necrotrophic phases of infection. Table 2 summarizes sequences of genes of pectate and pectin lyases identified in genomes of *Colletotrichum* sp.

7.2. Xylanases

Endoxylanases are produced by a number of saprophytic and pathogenic fungi [137, 138] and are responsible for the depolymerization of xylan in plant cell walls [139]. Over the past 20 years, a wide variety of xylanases have ben identified [140, 141] but so far the study of these enzymes has dealt mainly on the application in biotechnological purposes [142]. Only a few xylanases have been reported to be important factors of pathogenicity despite the fact that multiple xylanase genes have been identified in plant pathogenic fungi such as *C. carbonum, M. grisea, F. oxysporum* f. sp. *lycopersici, Helminthosporium turcicum,* and *B. cinerea* [141, 143-148]. In general, in some fungi where more than one gene has been identified for these enzymes, these are differentially expressed

depending on the substrate type or the invasion/nutritional stage in which they are examined [147].

The occurrence of several endoxylanases during infection has been a limiting factor in the generation of fungal mutants with reduced pathogenicity [143, 145, 149]. So far, no fungi with two or more disrupted xylanase genes have been reported. Considering the scarcity of studies on these depolymerases, the availability of sequenced genomes from *Colletotrichum* species will allow us to identify and analyze genes of putative endoxylanases of GH10 / GH11 families described in other fungi and their involvement in the infection process in their respective hosts (Table 2).

8. FUTURE DIRECTIONS

Although a significant amount of work dealing with virulence factors in *Colletotrichum* sp, has been published, particularly those related to their hemibiotrophic strategy of pathogenicity, many aspects remain to be solved. In general, the isolation and analyses of cDNAs and the use of specific mutants should allow to identify other virulence factors involved in the different phases of infection/invasion from biotrophy to necrotrophy. Understanding the role of these genes and how they are regulated is of paramount importance to get a clear insight into the mechanisms of plant-pathogen interactions. Currently, the use of data bases of sequenced genomes, transcriptomes and proteomes help to identify genes as those listed here; however, since not all genes were located in all genomes analyzed, one can assume that it may exist a group of general or basic virulence factors and probably others species-specific, probably related to virulence level, an idea supported by the identification of species-specific effectors by means of transcriptomes and secretomes from some species of the genus. These ideas may serve as a useful platform for future studies.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Capítulo 2

Se reporta el aislamiento, caracterización y el perfil de expresión genética del gen de la endo-β-(1,6)-D-galactanasa a partir de dos razas de *C. lindemuthianum* con distinto estilo de vida.

Differences in the expression profile of endo-β-(1,6)-D-galactanase in pathogenic and non-pathogenic races of *Colletotrichum lindemuthianum* grown in the presence of arabinogalactan, xylan or *Phaseolus vulgaris* cell walls

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A B S T R A C T

The scope of this study was the isolation and molecular characterization of endo-β-1,6-galactanase (*ebg*) from pathogenic (1472) and non-pathogenic (0) races of *Colletotrichum lindemuthianum* cultivated with arabinogalactan, xylan or *Phaseolus vulgaris* cell walls as carbon sources. Characterization of the *ebg* cDNA and 3D protein modeling revealed typical elements of the GH30 family of galactanases. The growth of both races with glucose showed basal transcription levels of *ebg*. When glucose was replaced with arabinogalactan, xylan or plant cell walls, *ebg* transcription markedly increased in race 1472 but not in race 0. Putative DNA-binding sites for Cre, Xlnr, ACEI, PacC and Gal4 transcriptional factors were predicted in *ebg* genes from *Colletotrichum* species.

1. Introduction

Colletotrichum lindemuthianum is an economically important hemibiotrophic phytopathogen. Along with its host, *Phaseolus vulgaris*, *C. lindemuthianum* represents a convenient model for studying the physiological and molecular basis of plant-pathogen interactions [1, 2]. *C. lindemuthianum* encompasses different strains or special forms known as races, physiological races or pathotypes. These pathotypes are classified based on their interactions with a group of 12 different *P. vulgaris* cultivars [3]. A non-pathogenic race and more than 100 pathotypes with different levels of virulence have been reported around the world. AFLP analyses of 10 of the 54 *C. lindemuthianum* pathotypes identified in México have revealed high genetic diversity and several lineages [3, 4]. During the biotrophic phase of fungal infection, a spore adheres to the plant surface and germinates to form a short germ tube, which differentiates into a melanized appresorium. This structure transforms internal pressure into mechanical force, allowing for penetration of the cuticle and epidermal cell wall (CW) and the formation of a vesicle and a primary hyphae within the cell [5]. A necrotrophic phase then follows, which begins 48-72 h after germination. This necrotic phase is characterized by the development of a secondary hyphae, which grows both intra- and intercellularly within plant tissues and secretes large amounts of various polysaccharidases that collectively degrade the host CW [5, 6]. Of the secreted polysaccharidases, pectinases [7-10] and xylanases [11] are thought to play a major role in pathogenesis. However, the role of other CW-degrading enzymes in the establishment of the infection is not well understood.

Endo-β-(1,6)-D-galactanase (EC 3.2.1.164) is a debranching hemicellulase that catalyzes the hydrolysis of β-(1,6)-galactosyl side chains in arabinogalactans (AGs), producing β-(1,6)-galacto-oligomers and β-(1,6)-galactobiose [12, 13]. Despite the critical role of endo-β-(1,6)-D-

galactanases in CW degradation, there have been few studies of these enzymes in fungi and bacteria, and so far their research has focused on the application in biotechnology. Accordingly, native endo-β-(1,6)-D-galactanases from *Aspergillus niger* and *Trichoderma viride* have been characterized [12, 13] and cDNAs from *T. viride* and *Neurospora crassa* have been sequenced, characterized and expressed in *Escherichia coli* and *Pichia pastoris,* respectively [14, 15]. There is only one report of an endo-β-(1,6)-D-galactanase in the actinomycete *Streptomyces avermitilis* [16]. Based on their amino acid sequences, endo-β-(1,6)-D-galactanases have been reassigned from the GH5 family to the GH30 family (Group 2, subgroup E) [17]. Proteins in the GH30 family possess a TIM-barrel fold structure [17, 18].

A useful strategy for exploring the role of CW-degrading enzymes in disease development is to search for differences between pathogenic and non-pathogenic races of the same fungal species. For instance, comparative analysis of gene expression and corresponding enzyme activities in pure cultures incubated in media containing different carbon sources can be a very informative approach. Using this strategy, we demonstrated that the expression of *Clpnl2* [19, 20] and *xyl1* (unpublished data), which code for pectin lyase and xylanase, respectively, are significantly different between pathogenic (1472) and non-pathogenic (0) races of *C. lindemuthianum* grown in the presence of different carbon substrates. Growth and production of extracellular pectin lyase differed between the pathogenic and non-pathogenic races of *C. lindemuthianum* as well. Such differences may be related to fungal pathogenesis.

Based on these results, we decided to extend our studies to other equally important plant cell wall depolymerases. Here, we report the isolation, characterization and expression analysis of *ebg,* which encodes an extracellular endo-β-1, 6-galactanase in pathogenic and non-pathogenic races of *C. lindemuthianum* cultivated in media containing different carbon sources. We also

present a 3D structure of the putative protein model generated via homology modeling of EBG from *C. lindemuthianum,* and clustal alignment of the deduced amino acid sequence. The putative DNA-binding sites for transcriptional factors that regulate genes encoding CW-degrading enzymes in *ebg* genes from *Colletotrichum s*pecies were also predicted. Finally, we carried out a phylogenetic analyses of genes identified in other species of the same genus.

2. Materials and methods

2.1 Strains and culture conditions

C. lindemuthianum, races 0 (non-pathogenic) and 1472 (pathogenic), were kindly provided by Dr. June Simpson (CINVESTAV-IPN, Unidad Irapuato, Mexico) and maintained on potato dextrose agar (PDA; Difco, México) at 28°C. For expression analysis, 1.6 mg (approximately 5 cm^2) of mycelia from both races was inoculated into 125 ml-Erlenmeyer flasks containing 50 ml of potato dextrose (PD) medium and shaken (150 rpm) at 28°C. After 10 days, mycelia were collected by filtration, washed with water and transferred to 125 ml-Erlenmeyer flasks containing 50 ml of modified Mathur's medium [21] supplemented with 2.5% of glucose, arabinogalactan (from larchwood; Sigma-Aldrich, St. Louis, MO, USA), xylan (from beechwood; Sigma-Aldrich, St. Louis, MO, USA) or CW from *P. vulgaris* (cv. Flor de Mayo) obtained from hypocotyls as described elsewhere [22]. Flasks were shaken (150 rpm) at 28ºC. After various periods of time, mycelia were collected by filtration, washed with water, frozen with liquid nitrogen and stored at -80ºC until use.

2.2 *DNA and RNA isolation*

Genomic DNA was isolated from *C. lindemuthianum* mycelia grown for 9 days in PD medium according to the protocol described for *Colletotrichum gloesporoides* [23]. Total RNA was

purified from mycelia using the TRIzol reagent (Invitrogen). RNA samples were treated with

DNAse I according to the manufacturer´s instructions (Invitrogen) to eliminate DNA. The

qualities and concentrations of total RNA extracts were assessed using a Biophotometer Plus

system (Eppendorf, Barkhausenweg, Hamburg, Germany).

2.3 *DNA and cDNA isolation, sequencing and analysis*

A cDNA fragment (1101 bp) of the *ebg* gene from *C. lindemuthianum* race 1472 was

amplified using the 3´RACE method with the SuperScript III First-Strand Synthesis System

(Invitrogen, Grand Island, NY, USA) as specified by the manufacturer. The degenerate upstream

primer BXILDeg1 5´-TTYGARTAYCCNGGN-3´, which was designed from conserved fungal

glycosyl hydrolase amino acid sequences reported in the National Center for Biotechnology

Information (NCBI) database, was used. Total RNA isolated from mycelia induced with xylan

for 24 h was used as the template. The resulting cDNA fragment was ligated into the pCR 4-

TOPO vector, cloned into *E. coli* TOP10 (TOPO TA cloning Kit, Invitrogen, Carlsband, CA,

USA), and 14 clones were sequenced (data not shown). The specific downstream primer ClebgR

(5´- ATGGACTGGTCGCTGAATAGG -3´) was designed from the *ebg* sequence determined in

this study. In contrast, the upstream primer ClebgD1 (5'-

CCCTAGTATGCAGCTCAAGTCTCT-3') was designed using the endo-β-(1,6)-galactanase

sequence from *Colletotrichum graminicola*, which is available in GenBank. The complete cDNA

and DNA of *ebg* from both fungal strains were amplified with the specific primers ClebgD1 and

ClebgR. The PCR mixture was heated to 94°C for 2 min in a thermocycler (Veriti Thermal

Cycler, Foster city, CA, USA), followed by 30 cycles of denaturation for 30 sec at 94°C,

annealing for 45 sec at 62°C, and extension for 30 sec at 72°C. A final extension step was

performed for 10 min at 72°C. PCR products were ligated into the pCR 4-TOPO vector, cloned

into *E. coli* TOP10, and 10 clones were sequenced.

DNA and cDNA sequences were determined for both strands via automated sequencing using

the dideoxy-chain termination method performed by Macrogen USA. Nucleotide sequences were

analyzed using Chromas Lite 2.1.1 (Technelysium Pty Ltd, Queensland, Australia). Multiple

sequence alignments were performed using the Clustal Omega program (European

Bioinformatics Institute) [24] and ClustalX [25]. Genomic nucleotide sequences of endo-β-(1,6)-

D-galactanase in *Colletotrichum* sp. were obtained from NCBI GenBank (Table 1). The sequence

of the N-terminal secretion signal was identified using the SignalP 4.1 Server [26], and protein

molecular masses and pI values were calculated using the Compute pI/Mw tool provided by the

ExPASy Proteomics Server (http://web.expasy.org/compute_pi/) [27]. Putative *N*-glycosylation

sites were predicted using the NGlycPred Server (http://exon.niaid.nih.gov/nglycpred/) [28], and

catalytic residues were predicted using the EXIA2 Server (http://203.64.84.196/index.php) [29].

Table 1. Endo-β-(1,6)-D-galactanase genes from *Colletotrichum* species and other fungi used for the analyses.

Specie	Amino acid number	Positives (%)	Identity (%)	Genome location	GenBank Access
C. orbiculare MAFF 240422 (A)	478	97	94	KB725912.1: 274415-275851	ENH83165.1
C. orbiculare MAFF 240422 (B)	481	82	68	KB726072.1: 732695-734140	ENH78243.1
C. incanum	481	89	80	LFIW01001173.1: 103068..104513	KZL83293.1
C. tofieldiae	481	89	81	LFIV01000068.1: 89853..91298	KZL71636.1
C. gloeosporioides Cg-14	477	88	79	AMYD01000698.1: 11769-13202	EQB56610.1

C. sublineola (A)	505	89	78	JMSE01000212.1: 16935-18452	KDN71284.1
C. sublineola (B)	491	78	67	JMSE01001303.1: 65966..67441	KDN62733.1|
C. fioriniae PJ7	481	88	79	JARH01000415.1: 358675..360120	XP_007595610.1
C. graminicola M1.001	482	87	76	GG697402.1: 123741..125189	EFQ35891.1
C.gloeosporioides Nara gc5	431	79	71	KB021271.1: 26129-26310, 26375-26726, 26777-27538	XP_007286566.1
C. higginsianum IMI 349063 (A)	481	83	71	CACQ02002444.1: 3870..5006	OBR13057.1
C. higginsianum IMI 349063 (B)	483	87	78	LTAN01000004.1: 4178546..4179997	OBR10869.1
C. salicis	481	88	78	JFFI01002404.1: 4840..6285	KXH34733.1
C. simmondsii	481	88	78	JFBX01000166.1: 363478..364923	KXH47435.1
C. nymphaeae SA-01	481	88	79	JEMN01001530.1: 196483..197928	KXH34302.1
Trichoderma viride	462	72	57		AB104898.1
Streptomyces avermitilis	491	64	51		BAC72917

2.4 *Protein modeling*

To identify potential functional and structural domains in *C. lindemuthianum* EBG, the online service Pfam (http://pfam.xfam.org/) [30] was used. We performed a computer analysis of protein secondary structures using the online services Jpred (http://www.compbio.dundee.ac.uk/jpred/) [31] and PROMALS3D Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/) [32]. The tertiary structure of the deduced *C. lindemuthianum* EBG amino acid sequence was predicted using an Iterative Threading ASSEmbly Refinement (I-TASSER) server and validated based on Confidence-score (C-score), TM-score and RMSD. [33]. Energy minimization of the model was performed by Anolea and GROMOS96, which were

provided by SPDBV 4.01 [34]. Molecular graphics and analyses were performed with the UCSF

Chimera package [35], SPDBV 4.01 [34] and RasMol 2.7.5 [36, 37].

2.5 *Expression analysis of ebg by qPCR*

Mycelia from *C. lindemuthianum* (race 0 and 1472) were grown with either 2.5% glucose for 8 h

or 2.5% arabinogalactan, xylan or CW from *P. vulgaris* for 0, 2, 4, 6, 12, 24 and 48 h and 3, 4, 5,

7 and 9 days as carbon substrate to induce *ebg* expression. Total RNA from 0.1 g of frozen

mycelia was isolated with the TRIzol reagent method and treated with DNAse I according to the

manufacturer´s instructions (Invitrogen) to eliminate DNA. The qualities and concentrations of

total RNA extracts were assessed using a Biophotometer Plus system (Eppendorf,

Barkhausenweg, Hamburg, Germany). Total RNA (1 μg) was used for cDNA synthesis

according to SuperScript III First-Strand Synthesis System kit (Invitrogen, Grand Island, NY,

USA).

A fragment of cDNA (137 bp) was amplified with the specific primers GalqD and GalqR (5'-

GACGGCGGTGGATGGGGAGTTA- 3'and 5'-TCCGCACCGCCGTCCAAGATAC- 3')

designed from *C. lindemuthianum ebg* gene (nt 1039-1060 and 1154-1175 respectively). The *C.*

lindemuthianum β-tubulin gene (*btub*) was used as reference gene. Fragments of cDNA (372 and

339 bp for races 1472 and 0, respectively) were amplified with the B36-F and B12-R (5´

CACCCACTCCCTCGGTGGTG 3´ and 5´CATGAAGAAGTGAAGACGCGGGAA 3´ primers,

respectively) [38] using total RNA obtained from mycelia grown with glucose. The β-tubulin

fragments were sequenced and deposited in GenBank (Accessions: KF487130 and KM587706).

The specific primers TR-βtub2-D and TR-βtub2-R (5´ GAATTCCCCGACC GTATGATG 3´

and 5´ CGGAGAGGGTGGCGTTGTA 3´, respectively) were designed and used as reference gen.

The specificity of the reaction was verified by a melting curve analysis and the efficiency of each primer was checked using the standard curve. Primers showed a slope of -3.153 and R^2 of 0.96 with an efficiency of 107.57%.

Reactions were carried out in a total volume of 10 μl, which contained 200 ng of cDNA, 2 pM of forward and reverse primers, 5 μl of EXPRESS SYBR GreenER qPCR Supermix Universal (Invitrogen, Carlsbad, CA, USA) and 25 μM of ROX Reference Dye (Invitrogen, Carlsbad, CA, USA). Controls without template were included in all real-time plates. The qPCR assays were performed in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems) and covered with MicroAmp Optical Adhesive Film (Applied Biosystems) on StepOnePlus Real-Rime PCR System (Applied Biosystems). Amplifications were performed under the following conditions: 95°C for 10 min, 40 cycles of 95°C for 40 s, 59°C for 40 s and 72°C for 40 s and a melt curve of 95ºC for 15 s, 60ºC for 1 min and 95ºC for 15 s.

Relative quantification of gene expression (qPCR) was performed using the comparative C_T method (ΔΔCt). Data were obtained from three independent experiments run in triplicate and analyzed in Excel. Analysis of variance (ANOVA) was carried out. The results are reported as the means with standard errors (SE). *P* values <0.05 were considered significant.

2.6 *Prediction of putative DNA binding sites for transcriptional factors*

The consensus DNA-binding sequences TATA box, initiator element (INR), downstream promoter element (DPE) and putative transcriptional start site (TSS) were located using the YAPP Eukaryotic Core Promoter program (http://www.bioinformatics.org/yapp/cgi-

bin/yapp.cgi). The CCAAT binding complex box [39], proximal DNA-binding elements for the carbon catabolite repressor CreA (SYGGRG) [40], and proximal DNA-binding elements for transcriptional activators controlling genes encoding xylanolytic and cellulolytic enzymes, Xlnr (GGCTAR) [41, 42], ACE I (AGGCA) [43], ACE II (GGSTAA) [44, 45], Pac C (GCCARG) [46], and Gal4 (CCGTT) [47] were located in *ebg* genes from *Colletotrichum* sp. (Table 1).

2.7 *Phylogenetic analyses*

Phylogenetic analyses were performed on the amino acid sequence deduced for *C. lindemuthianum* EBG (426 aa) and 15 endo-β-(1,6)-D-galactanase sequences from *Colletotrichum* sp. genomes. A sequence from the actinomycete *Streptomyces avermitilis* was included as outgroup (Table 1). Protein sequences were aligned with ClustalX [25] using default parameters. Prior to phylogenetic analyses, peptide signal sequences and N- and C-terminal extensions were excluded. Phylogenetic analysis was performed with the Bayesian Inference (BI) and Maximum likelihood criteria (ML) in MrBayes Vs. 3.2.2 [48] and RAxML (Randomized Axelerated Maximum Likelihood) version 8 [49]. Branch support values were estimated via a bootstrap analysis of 500 replicates and by posterior probabilities (PP). MrBayes runs were performed using the following parameters: amino WAG evolution model with gamma correction, four independent runs of four chains each (one cold chain and three hot chains) for 10 million generations with sampling every 1000 generations. Trees and parameters were summarized after discarding (burn-in) 25% of the data. The remaining trees were summarized as a majority consensus tree. Trees were visualized using FigTree v1.4.0 [50].

3.   Results and discussion

3.1 *Isolation and sequence analysis of ebg*

DNA and cDNA encoding an endo-β-(1,6)-D-galactanase were isolated from each race of *C. lindemuthianum* and deposited in GenBank (Accessions: KU587986, KU587987). No intron sequences were found in the *ebg* genes from either the pathogenic or non-pathogenic races. At the nucleotide and amino acid levels, the DNA and cDNA sequences from both races showed 100% identity. The *ebg* DNA and cDNA are 1440 bp in length with a 3′ UTR of 118 bp (Fig. 1). Comparison at the amino acid level with corresponding sequences in GenBank revealed a 94% to 67% identity with endo-β-1,6-galactanase genes in other *Colletotrichum* species and a 53% identity with the *T. viride* endo-β-1,6-galactanase (Table 1). A single gene without introns was found in *C. graminicola*, *C. simmondsii*, *C. salicis*, *C. nymphaeae, C. tofieldiae,* and *C. incanum. C. hingginsianum*, *C. sublineola,* and *C. orbiculare* contained two genes without introns that code for the same enzyme. In contrast, a gene without introns (strain Cg-14) and a gene with two introns (strain Nara gc5) were found in *C. gloesporioides*. The presence of one *ebg* gene in some *Colletotrichum* species is consistent with Southern blot analysis performed in *T. viride* [14].

The mature EBG protein is 460 amino acids in length. According to the SignalP 4.1 web server [26], the mature protein possesses a signal peptide cleavage site between Ala^{20} and Asp^{21}; which is consistent with the previously reported *T. viride* sequence [14]. According to the ExPASy Proteomics Server [27], the putative mature protein (residues 21 to 460) has a calculated molecular mass of 51070.64 Da and a pI of 5.3. These values are comparable to those for EBG from *A. niger*, *N. crassa* and *T. viride* [13-16]. Three putative *N*-glycosylation sites were predicted by the NGlycPred Server [28] at Asp^{45}, Asp^{233} and Asp^{416}, which are different from those reported for EBG from *T. viride* [14].

```
  1  M  H  F  S  T  L  A  A  V  A  V  S  L  L  V  A  P  I  N  A  D  T  T  T  T  I  E  A  K  S
     atgcacttctctaccctcgccgccgtggccgtgagcctcctggtagcacccatcaacgcggacacaaccacgaccatcgaagccaagtcc

 91  N  R  G  T  W  E  G  W  G  T  S  L  A  W  W  A  R  R  F  G  D  R  D  D  L  A  D  I  F  F
     aaccgcggcacgtgggaaggatggggcacctctctggcgtggtgggcacgacgattcggcgatcgcgatgacctcgccgacatcttcttc

181  T  T  N  S  T  T  W  S  G  T  S  L  P  G  L  G  L  N  I  V  R  H  N  A  G  A  S  S  F  N
     acgaccaactcgacaacctggtcgggcacctcgctacccggtttggggctcaacattgtccggcacaacgccggcgctagcagcttcaac

271  T  V  D  G  E  K  M  V  V  S  P  K  M  I  L  S  R  Q  I  E  G  H  W  V  D  W  Y  S  G  D
     acggtcgacggcgagaagatggtggtgtcgcccaagatgatactctcgcggcagattgaaggccactgggtcgactggtacagcggggac

361  P  T  S  T  S  W  K  W  D  V  D  T  N  Q  R  D  M  L  L  K  A  K  S  R  G  A  N  R  F  E
     ccgacatcgaccagctggaagtgggacgtggacaccaaccagcgcgatatgctgctcaaagccaaaagcagggggcgcgaaccgcttcgaa

451  L  F  S  N  S  P  M  W  W  M  T  K  N  H  N  P  S  G  S  A  D  G  T  E  N  I  Q  P  W  N
     ctttttcagcaactcgcccatgtggtggatgaccaagaaccacaacccctccggctccgcggacggcaccgagaacatccagccgtggaac

541  L  V  N  H  A  V  Y  M  A  T  V  A  K  Y  A  K  D  N  W  G  I  T  F  E  S  V  E  P  F  N
     cttgtcaatcacgccgtatacatggcaacggttgcgaaatacgcaaaggataattggggtataaccttcgagagcgtcgagcccttcaac

631 [E] P  S  A  S  W  W  T  A  D  G  T  Q  E  G  C  H  I  S  V  E  T  Q  S  T  L  I  A  A  L
     gaaccgagcgcgagctggtggacggcggacggcacgcaggagggctgccacatcagcgttgagacacaatccacccttatcgcggccttg

721  R  T  E  M  N  S  R  G  L  S  N  M  S  I  A  A  S  D  E  S  Y  Y  D  Q  A  V  A  T  F  K
     cggacggagatgaacagcagaggattgtcgaacatgagtatcgcagcttctgacgagtcgtactatgaccaggcggtggcgacgttcaaa

811  G  L  G  D  A  A  L  K  E  V  V  K  I  N  V  H  G  Y  Q  Y  G  N  G  A  R  A  S  L  H  E
     ggactcggcgatgcggctctcaaggaggtggttaaaatcaacgtccatggttaccagtacggcaacggcgcgcgcgcctcgcttcatgag

901  L  A  A  G  R  G  Q  R  V  W  N  S [E] Y  G  E  N  D  A  T  G  E  R  L  V  S  N  M  L  L
     ctggctgcgggccgcgggcagcgcgtgtggaactccgagtacggcgagaacgatgccacgggcgagaggctggtgagcaacatgttgctc

991  D  F  R  W  L  Q  P  Q  A  W  V  Y  W  Q  V  L  D  G  G  G  W  G  V  I  D  A  D  N  D  A
     gacttccgctggctacagcctcaggcgtgggtgtactggcaggtgctggacggcggtggatggggagttatcgacgcggacaacgacgct

1081 G  T  L  G  A  A  N  Q  K  Y  F  L  L  A  Q  F  A  R  H  I  R  E  G  M  R  I  L  D  G  G
     ggcacgctgggtgcagcgaaccagaagtacttttttgcttgctcagttcgcgcggcatattcgcgagggcatgcgtatcttggacggcggt

1171 A  D  N  V  V  A  A  Y  D  E  A  K  G  K  L  V  I  V  A  V  N  W  G  S  A  Q  Y  L  N  F
     gcggataacgtcgttgcggcgtacgacgaagcgaagggcaagttggtgattgtggcggtgaactggggcagcgcgcagtatctcaacttt

1261 D  L  G  K  F  S  Q  A  A  T  D  G  A  N  V  T  R  W  R  T  Q  I  G  S  G  D  R  Y  V  Q
     gacctaggcaagttctcgcaggcggcgacggacggtgcaaatgtcacaaggtggaggacgcaaattggcagtggcgaccgctacgtgcag

1351 A  Q  D  T  V  V  S  G  N  K  F  W  S  Y  F  E  S  K  M  V  Q  T  F  E  V  D  N  V  K  L
     gcgcaggacactgtcgtgagcgggaacaagttctggtcgtatttcgaaagcaagatggtgcagacattcgaggttgacaatgtcaagttg

1441 *
     tagcaggctggagcctggatggaacctttcctttccttttttttttcttcctccctcccctattcagcgaccagtccataattctttaa

1531
     gcagcacataacatcatcattcttgagtcgcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Fig. 1. Nucleotide sequence of the *ebg* gene and the deduced amino acid sequence. The underlined amino acid sequence denotes a fragment of the signal peptide. Boxed glutamic acids are putative catalytic residues found in the GH30 family. The double underlined DNA sequence corresponds to the 3' UTR.

Multiple sequence alignment of the amino acid sequence deduced for *C. lindemuthianum* EBG and amino acid sequences for endo-β-(1,6)-D-galactanases from *Colletotrichum* species and *T. viride* revealed conserved motifs (Fig. 2). The glutamic acid residues E^{211} and E^{312} found in the *T. viride* EGB sequence correspond to putative catalytic residues found in members of the GH5 family. However, EBGs were recently reassigned to the GH30 family. Analysis of the deduced amino acid sequence of *C. lindemuthianum* EBG revealed two Glu residues (E^{191}, E^{293}) in the mature protein that are conserved in *Colletotrichum* EBG sequences, with the exception of *C. gloesporoides* Nara-gc5, which lacks E^{191} (Fig 2). Although there is no experimental evidence that these residues correspond to catalytic residues in EBGs, the analysis performed by the EXIA2 Server http://203.64.84.196/index.php [29] confirmed the presence of these residues in the enzyme sequence.

3.2. *Protein homology modeling*

Homology modeling prediction of the 3D structure of EBG was performed with I-TASSER software, using a hierarchical method for protein structure prediction. Structures of a conserved exported protein from *Bacterioides fragilis* (PDB: 3CLW), a glycerol-bound, acid-β-glucosidase from *Homo sapiens* (PDB: 2NT0), the glucuronoxylan xylanohydrolase XynC from *Bacillus subtilis* (PDB: 3KL0) and SRFJ from *Salmonella typhimurium* were used as templates in I-TASSER. Five 3D models were generated. The EBG model with the most structural similarity to the templates had the following quality values: C-score of 0.22, TM-score of 0.74± 0.11 and a RMSD of 6.6± 4.0 Å (Fig. 3).
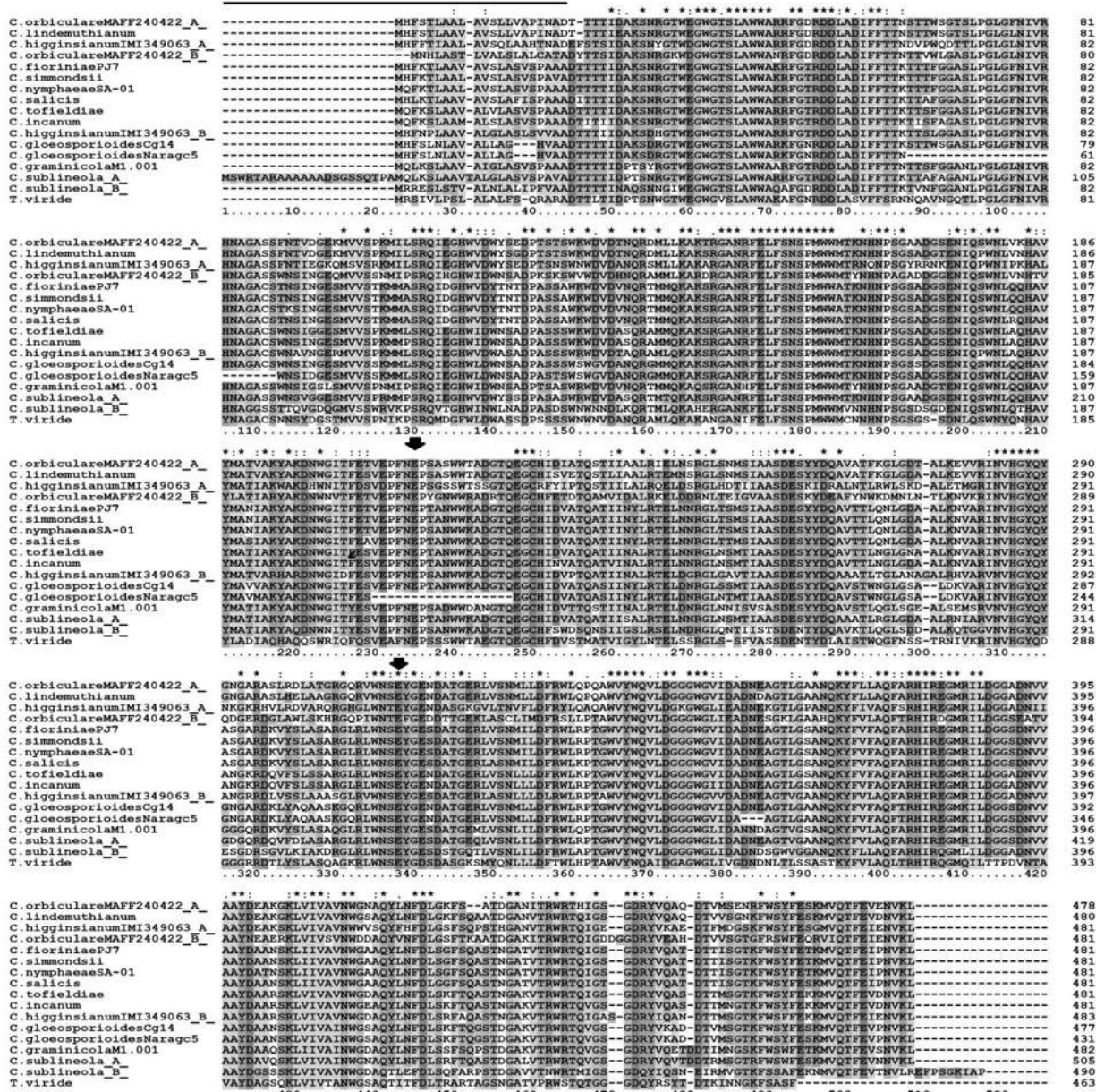
Fig. 2. Alignment of the endo-β-(1,6)-D-galactanase amino acid sequences from the *Colletotrichum* genus. Identical residues are marked with asterisks (*). Dashes represent gaps introduced to preserve alignment. Underlined residues correspond to the peptide signal. Conserved catalytic residues are indicated with black vertical arrows.
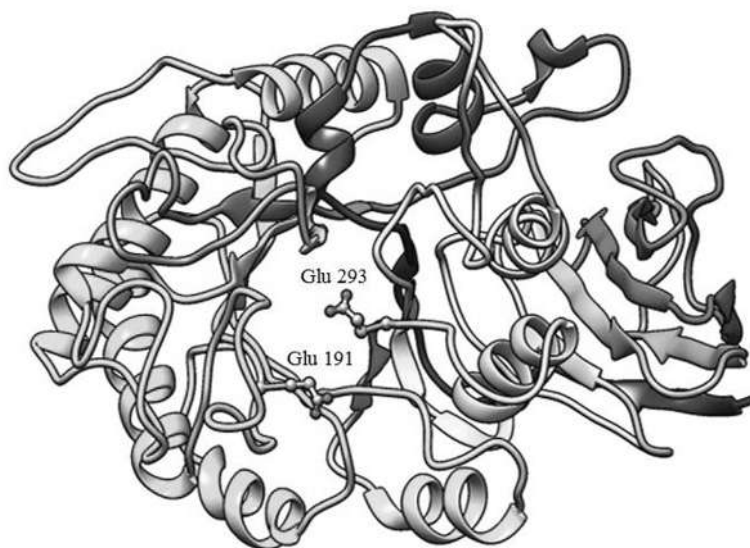
Fig. 3. Protein homology modeling. A 3D image of the tertiary structure of EBG from *C. lindemuthianum* generated by the I-TASSER server showing the TIM-barrel fold topology. The catalytic amino acids E^{191}, E^{293} are located on the enzyme surface.

Energy minimization of the protein model yielded a value of -22717.045 kJ/mol. A plot of dihedrals allowed the quality of the generated model to be assessed. The results were in agreement with the requirements for preferred and allowed regions, with the exception of 14 (3%) non-glycine residues. This model represents the first 3D model of an endo-β-(1,6)-D-galactanase.

The generated model exhibited a characteristic (β/α)₈ TIM-barrel fold topology. The canonical (β/α)₈-barrel consists of a closed eight-stranded parallel β-strand, forming a central barrel surrounded by eight α-helices. Active-site residues E^{191} and E^{293} are located on the catalytic face of the barrel, which consists of the C-terminal ends of the β-strands and the loops that link the β-strands with subsequent α-helices [51]. In addition, EBG possesses a side β-structure

characteristic of the GH30 family (Fig 3) whose function is not clear [17]. Some differences were observed between the canonical TIM-barrel and the predicted EBG model as this does not possess the characteristic $(\beta/\alpha)_8$ barrel showing only 7 β-strands in its central barrel.

To compare the 3D EBG model with hydrolases in the GH30 family, a structural alignment between EBG and the glucuronoxylan xylanohydrolase XynC from *B. subtilis* (PDB: 3KL0), one of the proteins used as a template in I-TASSER, was performed using the iPBA webserver (http://www.dsimb.inserm.fr/dsimb_tools/ipba/index.php) [52]. This analysis revealed that both enzymes possess the TIM-barrel fold topology; however, enzyme structures do not superimpose completely (Fig. 4A), probably due to differences in activity and ligand affinity that affect protein folding. EBG sequence showed 16% of identity and 22% of similarity with XynC. Nevertheless, a comparison of the predicted secondary structures for both enzymes using PROMALS3D revealed a similar α-helix/β-strand pattern in both proteins (Fig 4B). Using the online service Pfam (http://pfam.xfam.org/) [30], a Glyco_hydr_30_2 (O-Glycosyl hydrolase family 30) domain was identified spanning amino acid residues 3 to 250. These results support the hypothesis that EBG belongs to the GH30 family.

It has been reported that the acid catalyst/base E^{140} from XynC is highly conserved in the GH30 family, while the nucleophilic residue E^{229} is variable [17]. Protein alignment showed that the catalytic residues of EBG and XynC are conserved (Fig. 4B); however, the catalytic residues are located at different positions in each respective sequence: in XynC, the proton donor E^{140} is positioned in β-strand four whereas the catalytic nucleophile E^{229} is positioned in β-strand seven [17], while in EBG, the proton donor E^{191} is positioned in a coil and the catalytic nucleophile E^{293} is positioned in a β-strand.
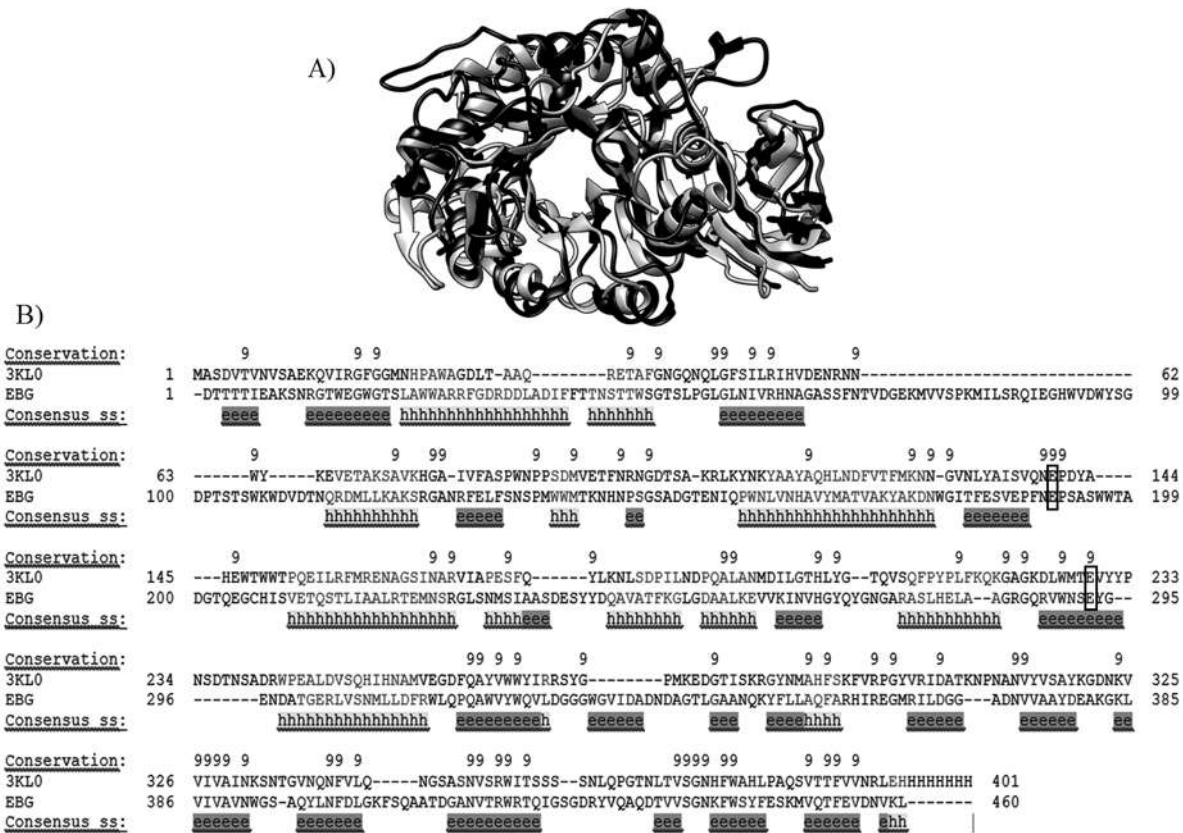
Fig. 4. Superimposition of 3D model structures for glucuronoxylan xylanohydrolase XynC from *B. subtilis* (PDB: 3KL0) (gray) and EBG (black) (A). (B), alignment of EBG and XynC sequences. The consensus secondary structure that generates β-strands (e) and α-helices (h) are indicated. The number 9 above the sequences indicates residues with greater conservation indices. Black squares indicate conserved catalytic residues. The structural alignment was generated using the iPBA web server.

3.3 *Expression analysis of ebg*

Induction of CW-degrading enzymes by different carbon substrates has been studied in some phytopathogenic fungi such as *Sclerotinia sclerotiorum* [53] *Sclerotium rolsfii* [54] and *Penicillium* sp. [55], among others. The involvement of some of these enzymes in the

development of bean anthracnose by *C. lindemuthianum* race γ was first described by

Wijesundera *et al.* [56]. Of the secreted polysaccharidases, pectinases [7-10] and xylanases [11]

are thought to play a significant role in pathogenesis. However, there are no studies on the role of

endo-β-(1,6)-D-galactanase in the onset of the infection.

Results presented here show a clear difference between the non-pathogenic (0) and pathogenic

(1472) races of *C. lindemuthianum* in terms of *ebg* transcript expression when they are grown

with different carbon substrates. When glucose was used as the main carbon source, basal

expression levels of the *C. lindemuthianum ebg* transcript were observed in both races,

suggesting catabolic repression [57] (Fig. 5). A basal production of xylanase and endoglucanase

[54], pectinases [53, 58] and cellulases [59, 60] has been described in the fungi *S. rolsfii, S.*

sclerotiorum, and *C. lindemuthianum*, respectively. In the models commonly studied such as

Aspergillus sp. and *Trichoderma* sp. the expression of hemicellulases is principally induced by

carbon sources [42, 61-63]. Accordingly, transcription is suppressed in the presence of D-glucose

and strongly induced in the absence of this monosaccharide and in the presence of hemicellulose

[64]. Our results are consistent with this observation. On this basis, we used the basal expression

with glucose for comparison with induction by arabinogalactan, xylan or CW fractions from *P.*

vulgaris.

When glucose was replaced with arabinogalactan, *ebg* transcription levels were increased in

both fungi. However, the expression profiles differed between the two races: thus, the pathogenic

race exhibited three main peaks of *ebg* expression after 2, 6 and 24 h of growth that were,

respectively 8-, 12- and 7.1-fold higher than that observed in glucose. In contrast, the non-

pathogenic race exhibited a single expression peak 3-fold higher than that observed in glucose
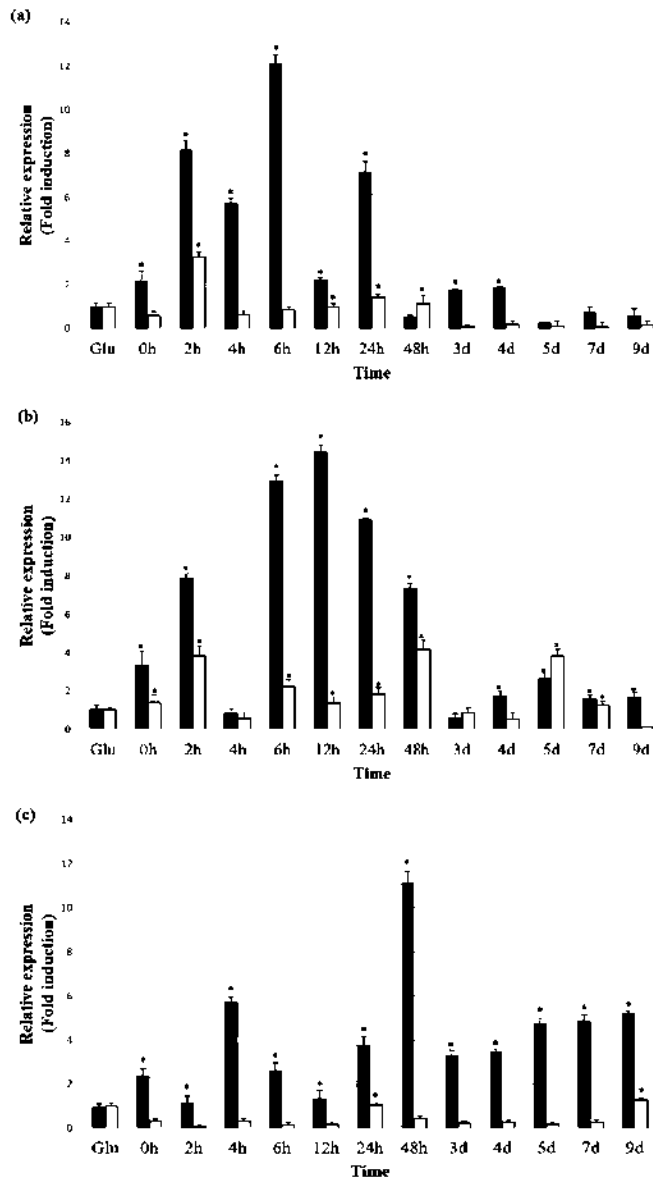
after 2 h of growth (Fig. 5a).

Fig. 5. Analyses of *ebg* expression in pathogenic (1472) and non-pathogenic (0) races of *C. lindemuthianum*. Expression analyses of *ebg* by RT-qPCR of cultures induced with arabinogalactan (a), xylan (b) and bean cell walls (c) are shown. Black bars, pathogenic race; white bars, non-pathogenic race. Each bar shows the mean of triplicates ± SE of three independent experiments. The symbol "*" indicates significant changes ($P<0.05$) in relation to the control (glu, glucose).

In the presence of xylan, the pathogenic race exhibited a high peak of *ebg* expression after 2 h of growth (8-fold higher with respect to glucose), which declined drastically after 4 h to 0.82-fold higher. Thereafter, expression increased over the following 6-48 h, reaching a maximum after 12 h (14.4-fold higher) and slowly decreasing to a low value (3-fold higher) over the following 3-9 days of incubation (Fig. 5b). In contrast, race 0 exhibited three peaks in *ebg* expression after 2 h, 48 h and 5 days (~4-fold higher) (Fig. 5b). It has been proposed that basal levels of endoxylanase commence degradation of xylan generating products that induce further enzymatic activity. In *A. niger*, xylose, as the final product of xylan degradation, functions as a regulator of the expression of xylanases, acting as an inducer at low concentrations and as a repressor at high levels [65-68]. However, xylose also induces expression of α-glucuronidase (*aguA*), acetylxylan esterase (*axeA*) and feruloyl esterase (*faeA*) and endoglucanase [42, 65, 69, 70]. Our results suggest that products of xylan degradation function as regulators of *ebg* expression in *C. lindemuthianum.*

The ability of CW fractions from *P. vulgaris* to induce *ebg* expression in *C. lindemuthianum* was also tested. As observed with arabinogalactan and xylan, two expression peaks were detected, which occurred after 4 and 48 h and were 5.7-, and 11-fold higher with respect to glucose, respectively. After 48 h, expression levels remained fairly constant for the following 3 to 9 days (~5-fold-higher). Interestingly, transcript levels in the non-pathogenic race were below basal values at all time points (Fig. 5c). The *ebg* expression profile observed in the pathogenic race over the first 48 h of growth in the presence of inducing carbon substrates agrees with the pattern of gene expression reported for other hydrolytic enzymes, such as pectin lyase 2 (*Clpnl2*) from *C. lindemuthianum* [20]. These findings indicate that products of arabinogalactan and xylan degradation and CW components can induce high levels of *ebg* expression in the pathogenic race but not in the non-pathogenic race. In fungi, the expression of extracellular hydrolytic enzymes is

coordinately regulated by transcriptional activators and repressors [70]. The expression of genes encoding xylanolytic enzymes is subject to catabolic repression through the action of CreA under a preferred carbon source and the activation through the action of XlnR under carbon limitation [57, 69, 71]. The transcriptional activator XlnR regulates genes encoding xylanolytic and cellulolytic enzymes and the use of D-xylose and L-arabinose via pentose catabolic pathway [72, 73]. XlnR homologs are found in the genomes of most filamentous ascomycetes and they are assumed to have the same function [72, 73].

To determine if in *ebg* genes from *Colletotrichum* genus exist putative DNA-binding sites for transcriptional activators and repressors of CW-degrading enzymes, we performed an analysis *in silico* for prediction of putative DNA-binding sites in 15 *ebg* genes from nine species of *Colletotrichum* (Table 1). We did not included analysis of the regulatory region of *ebg* from *C. lindemuthianum*, because not count with its sequences. Initially, the consensus DNA-binding sequences TATA box, initiator element (INR), downstream promoter element (DPE) and putative transcriptional start site (TSS) were located (Fig. 6, Table S1). Thus, the sequence corresponding to the UTR 5´ between the ATG and putative TSS varies between 564 to 772 bp. Seven genes have TATA box or TATA-like elements in their promoter, and eight exhibit a TATA-less core promoter. The DPE element was found in 11 genes, and the INR element was found in genes TATA-less or TATA-like (Table S1). The CCAAT binding complex box (Bc) identified in the promoters of many cellulase and hemicellulase genes is necessary for the full transcriptional activation of certain promoters [39]. Bc binding sites were located in 11 genes except for *C. orbiculare* (A), *C. higginsianum* (A) and *C. sublineola* (A) (Fig. 6, Table S1).

The analysis of prediction of DNA-binding sites it allowed the identification of proximal elements for Cre, Xlnr, ACEI, PacC and Gal4 transcriptional factors that regulate the expression of genes coding xylanolytic and cellulolytic enzymes (Fig. 6, Table S1). It has been reported that

there are two closely Cre binding sites in genes that suffer catabolic repression [70]; however, we identified at least four putative sequences in *ebg* from *C. fioriniae* PJ7 and up to 13 putative binding sites in *ebg* from *C. sublineola* (A). Xlnr binding sites were identified in most *ebg* sequences except in genes of *C. orbiculare* (B), *C. gloesporioides* Cg-14, *C. gloesporioides* Nara gc5, *C. tofieldiae,* and *C. incanum.* ACEI is a negative regulator of cellulases and xylanases in *T. reesei* [74]. ACEI binding sites (1 to 3) were found in the majority of the genes but were absent in *C. tofieldiae, C. incanum, C. graminicola* and *C. higginsianum* (B).
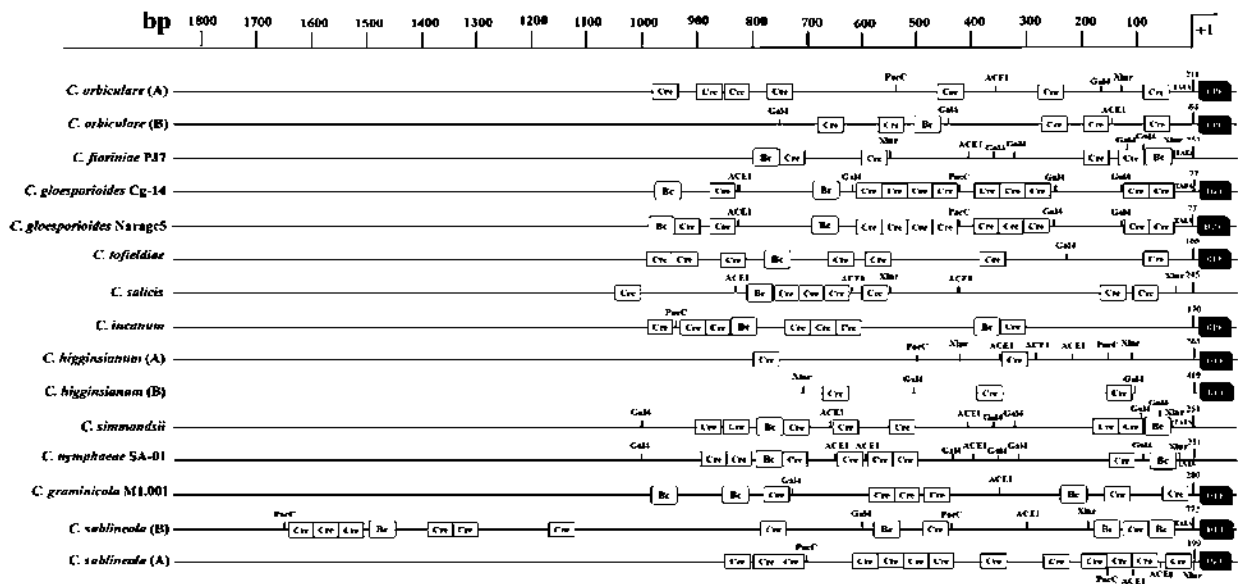

Fig 6. Putative DNA-binding sites for transcriptional factors that regulate genes coding CW-degrading enzymes, identified in *ebg* genes. TSS (transcriptional start site), number (+1) indicates the distance to the start codon of each sequence (ATG). DPE (downstream promoter element), Bc (CCAAT binding complex box). Putative DNA-binding sites for transcriptional factors Cre, Xlnr, ACEI, PacC, and Gal4 are indicated in each gene analyzed.

It was reported that ACEII is an essential universal activator controlling the transcription of cellulases and hemicellulases [75]. However, we failed to find ACEII binding sites in any gene. PacC is an activator for alkaline-expressed genes [70] and binding sites (1 to 2) for this regulator were found in seven genes. The binding sites for Gal4, a transcriptional factor that regulates galactose catabolism in yeast [57], were located in *ebg* genes except in *C. incanum*, *C. sublineola* A and *C. higginsianum* A. These results support the idea that *ebg* genes from *Colletotrichum* species are under the control of transcriptional factors that regulate the expression of genes coding xylanolytic and cellulolytic enzymes. However, further studies are necessary to confirm the function of the detected DNA consensus sequences and proximal elements and how *egb* genes are regulated.

A further question concerns the EBGs function beyond the generation of a carbon nutrient for the fungus. In plant CWs, arabinogalactan proteins (AGPs) are O-glycosylated at one or more hydroxyproline residues by type II arabinogalactans consisting of β-(1,3)- and β-(1,6)-galactan side chains connected to each other by (1,3)(1,6)-linked branch points. The O-3 and O-6 positions are substituted with terminal arabinosyl residues [76].

It has been demonstrated that AGP At3g4530 from *Arabidopsis thaliana* is covalently linked to CW hemicellulose and pectin, establishing a continuous network between CW carbohydrates and structural proteins [77]. Due to these linkages, AGPs are involved in several physiological processes, including plant-microbe interactions. AGPs act as soluble signals or as modulators and co-receptors in the apoplastic space. Thus, AGPs are considered by some authors as the first examples of mediators between the CW, the plasma membrane and the cytoplasm [76, 78].

There is presently no evidence supporting the role of arabinogalactan (AG)-degrading enzymes in the establishment of the infection. However, AGPs have been the subject of several

studies dealing with the mechanisms of plant-pathogen interactions. For instance, it has been observed that AGPs are accumulated at the interfaces between vascular tissue and lesions caused by fungi [79, 80].

The gene expression profile in the presence of β-Yariv, an arabinogalactan analog that binds to AGPs, was similar to the profile induced by plant wounds [81]. Moreover, the treatment of plant tissues with β-Yariv represses genes involved in gibberellin signaling, suggesting that AGPs are involved in the perception of signals that trigger defense responses [82].

Some AGPs have a conserved domain of six cysteine residues called a PAC domain (proline AGP cysteine domain). The PAC domain is anchored via a small glycoprotein, LAT 25, which interacts with a receptor like-kinase (RLK) known as LePRK. It has therefore been suggested that the PAC domain may serve as the connecting bridge between AGPs and RLKs [83].

AGPs are putative co-receptors in signaling pathways that function during growth and plant development [84]. RLK receptors are implicated in elicitor recognition and the triggering of plant defense mechanisms against phytopathogenic fungi. It has been demonstrated that the LysMRLK receptor exhibits high chitin affinity [85]. AGPs also play a key role in both beneficial and pathogenic root-microorganism interactions. They are essential for root cells to recognize beneficial microorganisms as well for root cells to trigger localized and efficient defense responses to control pathogenic organisms [86]. Since the carbohydrate groups in AGPs are critical for their function [76], it is conceivable to hypothesize that the pathogenic race of *C. lindemuthianum*, which more rapidly expresses *ebg* at higher levels in the presence of plant CW polysaccharides, is better adapted to degrade AGPs for the establishment of the infection.

3.4 *Phylogenetic analyses*

To elucidate the relationship between EBGs from both races of *C. lindemuthianum* and those from other *Colletotrichum* species, the amino acid sequence of *C. lindemuthianum* EBG and 15 other EBG sequences from nine species were analyzed phylogenetically. The EBG sequence of the actinomycete *Streptomyces avermitilis* was used as outgroup (Table 1). The results showed a separation of EBGs into three clades (Fig. 6). In clade I the galactanase from *C. lindemuthianum* was grouped with the EBGs from *C. orbiculare* A and B, the nearest species according to a phylogenetic analysis of *Colletotrichum* sp. [87, 88], and *C. higginsiamun* A. Clade II showed an additional clustering into two subclades. One subclade included EBGs from *C. nymphaeae, C. salicis, C. simmondsii*, and *C. fioriniae,* which infect dicots. On the other hand, *C. graminicola*, *C. higginsianun* B, *C. sublineola* A and B, *C. tofieldiae*, and *C. incanum* were included in a second subclade. Finally, in clade III the EBGs from *C. gloesporioides,* a pathogen of dicots were grouped.

There are no previous reports regarding phylogenetic analyses of endo-β-(1,6)-D-galactanases. At this point, it is worth noting that our results do not show a diversification of EBGs, yet there is a separation of proteins into different groups or lineages. It has been observed that pathogenic fungi of monocot and dicot plants are better adapted to correspondingly degrade the CW of monocot and dicot plants clearly reflecting a host preference [89]. Transcriptome analyses of *C. graminicola* and *C. higginsianum* during interactions with monocots and dicots, which exhibit different CW compositions, have revealed differences in the expression levels of polysaccharidases. In the case of *C. higginsianum,* such differences allow the organism to invade a broader range of host plants [90]. In our analysis, although two groups or lineages of EBGs included pathogens of dicot plants (Clades I and III), one lineage of EBGs also included

pathogens of monocot and dicot plants, an entomopathogen, and an endophyte suggesting that evolution is not necessarily influenced by the host preference.
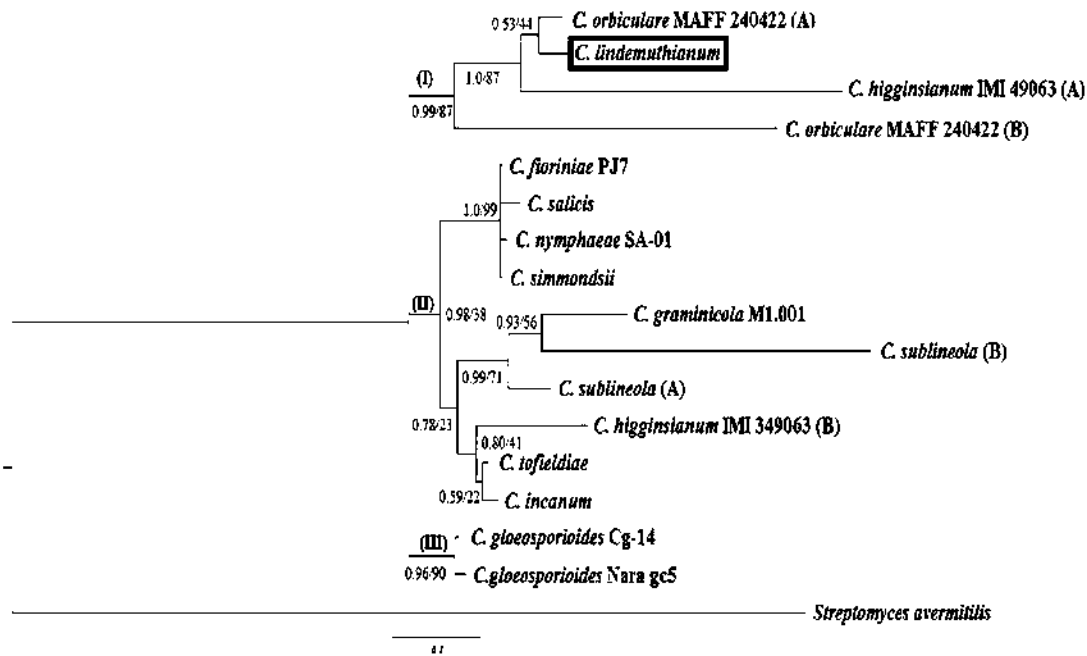

Fig. 7. Phylogenetic analyses of endo-β-(1,6)-D-galactanases from *Colletotrichum* species. The phylogenetic tree was constructed using the ML and BI methods. The general topology obtained is represented by the Bayesian 50% majority rule consensus tree. BI posterior probabilities and ML bootstrap support are indicated on the branches. The numbers at the nodes indicate bootstrap values based on 1,000 bootstrap replications.

4.   Conclusion

To our knowledge, this is the first report of cDNA isolation, 3D protein modeling, phylogenetic and expression analyses of endo-β-(1,6)-D-galactanases, a GH30 family protein, from *C. lindemuthianum*. The study revealed different *ebg* expression profiles between pathogenic and non-pathogenic races of *C. lindemuthianum,* suggesting a role of this enzyme in

the degradation of plant CW during the onset of infection. In addition to the putative consensus

DNA-binding sequences TATA box, INR, DPE and TSS in 15 *ebg* genes from nine species of

Colletotrichum, we also found putative DNA-binding sites for the transcriptional factors Cre,

Xlnr, ACEI, PacC and Gal4, which regulate the expression of genes coding CW-degrading

enzymes. Finally, phylogenetic analysis of EBGs from a number of *Colletotrichum* species

revealed three protein lineages.

Acknowledgements

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Supplementary material

Table S1. Consensus DNA-binding sequences and putative DNA-binding sites for transcriptional factors located in *ebg* genes from *Colletotrichum* species.

Specie	TSS	DPE	INR	TATA	Binding Complex	Cre	Xnlr	ACE I	ACE II	PacC	Gal4
C.orbiculare (A)	211	AGTTA (+28/+32)		TTGATATAGACG (-37/-26)		GCGGG (-93/-89) (-437/-433) (-968/-964) CTGAG (-236/-232) CCGAG (-776/-772) (-900/-896) GCGAG (-840/-836)	GGCTAG (-153/-148)	AGGCA (-383/-379)		GCCAAG (-526/-521)	CCGTT (-174/-170)
C.orbiculare (B)	64	AGATC (+28/+32)	CCATTTC (+1/+6)		CCAAT (-501/-497)	GCGGG (-93/-89) (-516/-512) (-646/-642) (-669/-665) CCGAG (-200/-196) (-258/-254)		AGGCA (-171/-167)			CCGTT (-448/-444) (-776/-772)
C. fioriniae PJ7	252		CCATTCT +2/+8	TGAATAAAACCG -20/-9	CCAAT (-44/-40) (-766/-762)	CCGAG (-92/-88) GCGGG (-170/-166) (-597/-593) (-749/-745)	GGCTAA (-34/-29) GGCTAG (-583/-578)	AGGCA (-402/-398)			CCGTT (-60/-56) (-85/-81) (-356/-352) (-378/-374)
C.gloeosporioides Cg-14	77	GGATC (+28/+32)	TCAATAT (+2/+8)	GACATAAAGGTC (-31/-20)	CCAAT (-676/-672) (-957/-953)	GCGGG (-70/-66) (-319/-315) (-374/-370) CCGAG (-86/-82) (-281/-277) (-574/-570) CCGGG (-513/-509) CTGAG (-534/-530) (-541/-537) GCGAG (-946/-942)		AGGCA (-821/-817)		GCCAAG (-452/-447)	CCGTT (-126/-124) (-257/-253) (-622/-618)
C. gloeosporioides Naragc5	77	GGACT (+28/+32)	CCAATAT (+2/+8)	GACATAAAGGTC (-31/-20)	CCAAT (-682/-678) (-963/-959)	GCGGG (-70/-66) (-320/-316) (-375/-371) CCGAG (-86/-82) (-282/-278) (-575/-571) (-844/-840) CCGGG (-514/-510) CTGAG (-535/-		AGGCA (-827/-823)		GCCAAG (-453/-448)	CCGTT (-126/-122) (-258/-254)

Species										
						531) (-542/-538) GCGAG (-952/-948)				
C .tofieldiae	166	AGTCA (+28/+32)	CCACACC (+1/+6)		CCAAT (-740/-736)	CTGGG (-72/-68) (-610/-606) GTGGG (-373/-369) (-583/-579) (-973/-969) CTGAG (-815/-811) GCGGG (-916/-912)				CCGTT (-220/-216)
C.salicis	245		CCAAACT (-3/+3)		CCAAT (-775/-771)	CCGAG (-97/-93) (-640/-636) GCGGG (-175/-171) (-599/-595) CTGGG (-635/-631) (-1015/-1011) GCGAG (-758/-754)	GGCTAA (-39/-34) GGCTAG (-585/-580)	AGGCA (-409/-405) (-623/-619) (-806/-802)		CCGTT (-65/-61
C. incanum	170	AGTCA (+28/+32)	CCACACC (+1/+6)		CCAAT (-390/-386) (-801/-797)	GTGGG (-375/-371) GCGGG (-618/-614) (-976/-972) CCGGG (-666/-662) CCGAG (-682/-678) GCGAG (-827/-823) CTGAG (-876/-872)			GCCAAG (-952/-947)	
C. higginsianum (A)	263	AGTCA (+28/+32)	TCACTAG (+1/+6)			GCGGG (-308/-304) GCGAG (-788/-784)	GGCTAA (-119/-114) (-424/-419)	AGGCA (-227/-223) (-290/-286) (-332/-328)	GCCAAG (-128/-123) (-497/-492)	
C. higginsianum (B)	419	GGATC (+28/+32)	TTAATCT (-1/+6)			GCGGG (-131/-127) GCGAG (-374/-370) CTGAG (-683/-679)	GGCTAA (-722/-717)			CCGTT (-117/-113) (-502/-498)
C. simmondsii	251		TCATTCT (+2/+8)	TGAATAAAACCG (-20/-9)	CCAAT (-44/-40) (-770/-766)	CCGAG (-92/-88) (-896/-892) GCGGG (-170/-166) (-514/-510) (-600/-596) (-753/-749) CCGGG (-812/-808)	GGCTAA (-34/-29)	AGGCA (-402/-398) (-624/-620)		CCGTT (-60/-56) (-85/-81) (-356/-352) (-378/-374) (-1023/-1019)
C. nymphaeae	251		CCATTCT (+2/+8)	GAATAAAACCG (-19/-9)	CCAAT (-44/-40)	GCGGG (-170/-	GGCTAA (-34/-29)	AGGCA (-402/-		CCGTT (-60/-56)

					(-767/-763)	166) (-514/-510) (-597/-593) (-750/-746) CCGAG (-640/-636) (-893/-889) CCGGG (-809/-805)	398) (-579/-575) (-621/-617)			(-356/-352) (-378/-374) (-438/-434) (-1013/-1009)
C. graminicola M1.001	280	GGTCC (+28/+32)	CCACTAC (-1/+5)		CCAAT (-265/-261) (-810/-806) (-991/-987)	GTGGG (-15/-11) (-778/-774) GCGAG (-166/-162) (-552/-548) GCGGG (-493/-489) CTGAG (-546/-542)	GGCTAG (-346/-341)			CCGTT (-752/-748)
C. sublineola (B)	772	AGTCG (+26/+30)	CCAATCT (+2/+8)	TGAATAAATCTC (-33/-22)	CCAAT (-45/-41) (-124/-120) (-573/-569) (-1469/-1465)	GCGAG (-56/-52) CTGGG (-485/-481) CTGAG (-779/-775) (-1127/-1123) (-1302/-1298) GTGGG (-1333/-1329) GCGGG (-1513/-1509) GTGAG (-1525/-1521) CCGAG (-1542/-1538)	GGCTAA (-170/-166)	AGGCA (-306/-302)	GCCAAG (-467/-462) (-1590/-1585)	CCGTT (-608/-604)
C. sublineola (A)	199	GGTCA (+27/+31)	TTAGTTC (+1/+6)			GCGAG (-28/-24) (-156/-152) (-551/-547) (-714/-710) GTGGG (-127/-123) (-517/-513) CTGGG (-131-127) (-383/-379) CCGGG (-248/-244) GCGGG (-495/-491) CCGAG (-597/-503) (-779/-775) (-841/-837)	GGCTAA (-6/-1)	AGGCA (-35/-31) (-136/-132)	GCCAGG (-170/-165) (-719/-715)	

Capítulo 3

Artículo de Investigación que será sometido a la revista PHYSIOLOGICAL AND
MOLECULAR PLANT PATHOLOGY.

Se reporta el aislamiento, caracterización y el perfil de expresión genética del gen de la α-L-
arabinofuranosidasa a partir de dos razas de *C. lindemuthianum* con distinto estilo de vida.

Differences in the expression profile of a GH51 α-L-arabinofuranosidase in pathogenic and non-pathogenic races of *Colletotrichum lindemuthianum* grown in the presence of arabinogalactan, xylan or *Phaseolus vulgaris* cell walls

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A B S T R A C T

The scope of this study was the isolation and molecular characterization of GH51 α-L-arabinofuranosidase (*abfA*) from pathogenic (1472) and non-pathogenic (0) races of *Colletotrichum lindemuthianum* cultivated with arabinogalactan xylan or *Phaseolus vulgaris* cell walls as carbon sources. Characterization of the *abfA* cDNA and 3D protein modeling revealed typical elements of the GH51 family of arabinofuranosidases. The growth of both races with glucose showed basal transcription levels of *abfA*. When glucose was replaced with arabinogalactan, xylan or plant cell walls, *abfA* transcription markedly increased in race 1472 but not in race 0. Putative DNA-binding sites for Cre, Xlnr, ACEI, PacC, AreA and Gal4 transcriptional factors were identified by prediction in *abfA* genes from *Colletotrichum* species.

1. Introduction

Colletotrichum lindemuthianum is an economically important hemibiotrophic phytopathogen. Along with its host, *Phaseolus vulgaris*, *C. lindemuthianum* represents a convenient model for studying the physiological and molecular basis of plant-pathogen interactions [1, 2]. *C. lindemuthianum* encompasses different strains or special forms known as races, physiological races or pathotypes. These pathotypes are classified based on their interactions with a group of 12 different *P. vulgaris* cultivars [3]. A non-pathogenic race and more than 100 pathotypes with different levels of virulence have been reported around the world. AFLP analyses of 10 of the 54 *C. lindemuthianum* pathotypes identified in México have found high genetic diversity and several lineages [3, 4]. During the biotrophic phase of fungal infection, a spore adheres to the plant surface and germinates to form a short germ tube, which differentiates into a melanized appresorium. This structure transforms internal pressure into mechanical force, allowing for penetration of the cuticle and epidermal cell wall (CW) and the formation of a vesicle and a primary hyphae within the cell [5]. A necrotrophic phase then follows, which begins 48-72 h after germination. This necrotic phase is characterized by the development of a secondary hyphae, which grows both intra- and intercellularly within plant tissues and secretes large amounts of various polysaccharidases that collectively degrade the host CW [5, 6]. Of the secreted polysaccharidases, pectinases [7-10] and xylanases [11] are thought to play a major role in pathogenesis. However, the role of other CW-degrading enzymes in the establishment of the infection is unknown.

The α-L-arabinofuranosidases (ABF) (EC3.2.1.55) are hydrolytic cell wall exo type enzymes which catalyzes the hydrolysis of arabinosyl branches attached through α-L-(1,2), α-L-(1.3), α-L-(1,5) O-glycosidic anchors to arabinoxylans, arabinoxilooligosaccharides, arabinan,

arabinogalactans and arabino-oligosaccharides [12-14]. This polysaccharidases are classified in GHs 2,3,10, 43, 51, 54, 62 based in their amino acid sequence according to Carbohydrate-Active enZYmes database (CAZY). Recently, degradation regions-selectivity of GH51 and GH54 ABFs were studied, GH51 ABFs removed arabinosyl residues from internally and terminal non-reducing end of xylopyranosyl residues with mono and di-substitutions (m/d), showing ABF-m/d activity and demonstrating versatility in substrate specificity [15].

ABFs act synergistically with xylanases and β-xylosidases in arabinoxylan depolymerization [16, 17]; on the other hand, a synergistic effect of α-L-arabinofuranosidase, exo-β-(1,3)-D-galactanase and endo-β-(1,6)-D-galactanase is required for arabinogalactans type II complete degradation [18, 19]. Thus, ABFs have been applied in different agro-industrial processes: arabinose production as anti-glycemic agent, anti-metastatic and anti-carcinogenic compounds production, wine industry, acetic acid production and quality of the bread, improvement of animal feedstock, pulp and paper industry, juice industry, fermentable sugars production for bioethanol industry and synthesis of pentose-containing compounds [20]. Because to potential biotechnological applications, some coding genes of GH51 ABFs have been cloning and sequenced from fungi: *Aspergillus niger* N402 and ATCC 120120 [21-23], *Aspergillus kawachii* and *Aspergillus awamori* [24], *Aspergillus oryzae* [25], *Penicillium purpurogenum* [26], *Pleorotus ostreatus* [27] and *Penicillium chrysogernum* [28].

A useful strategy for exploring the role of CW-degrading enzymes in disease development is to search for differences between pathogenic and non-pathogenic races of the same fungal species. For instance, comparative analysis of gene expression and corresponding enzyme activities in pure cultures incubated in media containing different carbon sources is an informative approach. Using this strategy, we demonstrated that the expression of *Clpnl2* [29,

30], *xyl1* [31] and *ebg* [32] which code for pectin lyase, xylanase and endo-β-(1,6)-D-galactanase respectively, are significantly different between pathogenic (1472) and non-pathogenic races of *C. lindemuthianum* grown in the presence of different carbon substrates. Growth and production of extracellular pectin lyase differed between the pathogenic and non-pathogenic races of *C. lindemuthianum* as well. Such differences may relate to fungal pathogenesis.

Based on these results, we decided to extend our studies to other equally important plant cell wall depolymerases. Here, we report the isolation, characterization and expression analysis of *abfA*, which encodes an extracellular α-L-arabinofuranosidase (ABFA). We present a 3D structure of the putative protein model generated via homology modeling of ABFA from *C. lindemuthianum,* and clustal alignment of the deduced amino acid sequence. We analyze *abfA* expression in pathogenic and non-pathogenic races of *C. lindemuthianum* cultivated in media containing different carbon sources. We also made the prediction of putative DNA-binding sites for transcriptional factors that regulate genes encoding CW-degrading enzymes, in *abfA* genes from *Colletotrichum s*pecies. Finally, we present phylogenetic analyses of genes identified in other species of the same genus.

2. Materials and methods

2.2 Strains and culture conditions

C. lindemuthianum races 0 (non-pathogenic) and 1472 (pathogenic) were kindly provided by Dr. June Simpson (CINVESTAV-IPN, Unidad Irapuato, Mexico) and maintained on potato dextrose agar (PDA; Difco, México) at 28°C. For expression analysis, 1.6 mg (approximately 5 cm^2) of mycelia from both races was inoculated into 125 ml-Erlenmeyer flasks containing 50 ml of potato dextrose (PD) medium and shaken (150 rpm) at 28°C. After 10 days, mycelia were

collected by filtration, washed with water and transferred to 125 ml-Erlenmeyer flasks containing 50 ml of modified Mathur's medium [33] supplemented with 2.5% of glucose, arabinogalactan (from larchwood; Sigma-Aldrich, St. Louis, MO, USA), xylan (from beechwood; Sigma-Aldrich, St. Louis, MO, USA) or CW from *P. vulgaris* (cv. Flor de Mayo) obtained from hypocotyls as described elsewhere [34]. Flasks were shaken (150 rpm) at 28ºC. After various periods of time, mycelia were collected by filtration, washed with water, frozen with liquid nitrogen and stored at -80ºC until use.

2.2 *DNA isolation*

Genomic DNA was isolated from *C. lindemuthianum* mycelia grown for 9 days in PD medium according to the protocol described for *Colletotrichum gloesporoides* [35].

2.3 *abfA gene cloning, sequencing and analysis*

The complete DNA of *abfA* gene from both fungal strains was amplified with the specific primers ClabfAutrD and ClabfutrR2 (5'-TAGGTAGACAGGAAGCAAAGATGG- 3´and 5'-AACCGTTTTGCTCGACTATGGAC- 3´) designed from *abfA* gene located in the genome of *C. orbiculare* (Table 1). The PCR mixture was heated to 94°C for 5 min in a thermocycler (Veriti Thermal Cycler, Foster city, CA, USA), followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 45 sec at 63°C, and extension for 30 sec at 72°C. A final extension step was performed for 10 min at 72°C. PCR products were ligated into the pCR 4-TOPO vector, cloned into *E. coli* TOP10, and 12 clones of each race were sequenced.

DNA sequences were determined for both strands via automated sequencing using the dideoxy-chain termination method performed by Macrogen USA. Nucleotide sequences were analyzed using Chromas Lite 2.1.1 (Technelysium Pty Ltd, Queensland, Australia) and CLC

Sequence Viewer 7.8.1 (QIAGEN Aarhus A/S). Introns and exons prediction were carried out with Augustus (gene prediction) [36]. Multiple sequence alignments were performed using the Clustal Omega program (European Bioinformatics Institute) [37], ClustalX [38] and CLC Main Workbrench 7.8.1 (QIAGEN Aarhus A/S). Genomic nucleotide sequences of α-L-arabinofuranosidase in *Colletotrichum* sp. were obtained from NCBI GenBank (Table 1). The sequence of the N-terminal secretion signal was identified using the SignalP 4.1 Server [39], and protein molecular masses and pI values were calculated using the Compute pI/Mw tool provided by the ExPASy Proteomics Server (http://web.expasy.org/compute_pi/) [40]. Putative *N*-glycosylation and *O*-glycosylation sites were predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) [41] respectively.

Table 1. α-L-arabinofuranosidase genes from *Colletotrichum* species and other fungi used for the analyses.

Specie	Amino acid number	Positives (%)	Identities (%)	Genome location	GenBank Access
C. lindemuthianum 89	665	100	99	MASP02000226: 78676-78801 78860-79321 79384-79500 79557-79745 79809-80912	
C. orbiculare MAFF 240422	818	98	98	KB725740.1: 218099-218224, 218278-218739, 218797-218913, 218972-219161, 219219-220317, 220643-221105	ENH86152
C. gloeosporioides Cg-14	663	93	88	AMYD01003955.1: 9096..10961, 11021..11146	EQB44584

C.gloeosporioides Nara gc5	663	93	88	KB020814.1: 91472-91597, 91657-93522	ELA30166
C. chlorophyti	662	92	87	MPGH01000011.1: 460698-461981, 462038-462154, 462212-462673, 462736-462861	OLN97176
C. tofieldiae CBS 130851	663	91	85	LFIV01000227.1: 5634-6920, 6977-7093, 7148-7609, 7671-7796	KZL65318
C. higginsianum IMI 349063	686	92	87	LTAN01000008.1: 1371785-1373071, 1373129-1373245, 1373302-1373763, 1373825-1374019	XP_018153191 OBR04673
C. salicis CBS 607.94	683	89	83	JFFI01001763.1: 36319-37605, 37661-37777, 37836-38483	KXH54816
C. sublineola	664	91	84	JMSE01001044.1: 65435-65560, 65634-66095, 66158-66274, 66341-67630	KDN65327
C. simmondsii	663	90	85	JFBX01000594.1: 524722-526008, 526070-526186, 526245-526706, 526768-526893	KXH34038
C. nymphaeae SA-01	663	91	85	JEMN01000994.1: 70184-70309, 70372-70833, 70892-71008, 71064-72350	KXH52946
C. incanum MAFF238704	663	91	85	KV841934.1: 135849-135974, 136036-136497, 136552-136668, 136725-138011	OHW96222
C. fioriniae PJ7	663	91	85	JARH01000254.1: 3327-4613, 4675-4791, 4850-5311, 5373-5498	XP_007593005 EXF83319
C. orchidophilum IMI 309357	663	90	84	MJBS01000049.1: 89624-89749, 89809-90270, 90329-90445, 90501-91787	OHE98152

C. graminicola M1.001	663	90	83	GG697354.1: 325433-325558, 325642-326103, 326161-326277, 326340-327626	XP_008095314 EFQ31294
C. acutatum strain 1 KC05_12	663	90	85	LUXP01000011: 1156620-1156634, 1156696-1157157, 1157216-1157332, 1157394-1158680	
C. godetiae C184	663	90	85	LZRM01001484.1: 33066-34352, 34408-34524, 34584-35045, 35105-35230	
Aspergillus kawachii	628	53	38		BAB96815
Pleorotus ostreatus Type Florida	646	49	35		CCC33069

2.4 *Protein modeling*

To identify potential functional and structural domains in *C. lindemuthianum* ABFA, online service Pfam (http://pfam.xfam.org/) [42] was used. We performed a computer analysis of protein secondary structures using CLC Main Workbench 7.8.1 (QIAGEN Aarhus A/S). The tertiary structure of the deduced *C. lindemuthianum* ABFA amino acid sequence was predicted using an Iterative Threading ASSEmbly Refinement (I-TASSER) server and validated based on Confidence-score (C-score), TM-score and RMSD [43]. Structure refinement was performed with ModRefiner algorithm [44] and energy minimization of the model was performed by Anolea and GROMOS96, which were provided by SPDBV 4.01 [45]. Molecular graphics and analyses were performed with the UCSF Chimera package [46] and SPDBV 4.01 [45].

2.5 *Expression analysis (qPCR) of abfA*

Mycelia from *C. lindemuthianum* (race 0 and 1472) were grown with 2.5% of glucose for 8 h or with 2.5% of arabinogalactan, xylan or CW from *P. vulgaris* for 0, 2, 4, 6, 12, 24 and 48 h and 3, 4, 5, 7 and 9 days as carbon source to induce *abfA* expression. Total RNA from 0.1 g of frozen mycelia was isolated with TRIzol Reagent method and treated with DNAse I according to the manufacturer´s instructions (Invitrogen) to eliminate DNA. The qualities and concentrations of total RNA extracts were assessed using a NanoDrop One (ThermoFisher Scientific). Total RNA (1 µg) was used for cDNA synthesis according to SuperScript III First-Strand Synthesis System kit (Invitrogen, Grand Island, NY, USA).

A fragment of cDNA (137 bp) was amplified with the specific primers AbfART-D and AbfART-R (5'-CCTCGGCTGGACCTCGTACAAG- 3'and 5'-GGGTGCGGTAGGGGTGGTAGTC- 3') designed from *C. lindemuthianum abf* gene (nt 1343-1364 and nt 1485-1506 respectively). The *C. lindemuthianum* β-tubulin gene (*btub*) was used as reference gene. Specific primers TR-βtub2-D and TR-βtub2-R (5´ GAATTCCCCGACC GTATGATG 3´ and 5´ CGGAGAGGGTGGCGTTGTA 3´, respectively), reported to *C. lindemuthianum* were used [31].

The specificity of the reaction was verified by a melting curve analysis and the efficiency of each primer was checked using the standard curve method. Primers showed a slope of -3.153 and R^2 of 0.96 with an efficiency of 107.57%.

Reactions were carried out in a total volume of 10 µl, which contained 200 ng of cDNA, 0.2 pM of forward and reverse primers, 5 µl of Fast SYBR Green Master Mix (2X) (Applied Biosystems, USA). Controls without template were included in all real-time plates. The qPCR assays were performed in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems) and covered with MicroAmp Optical Adhesive Film (Applied Biosystems) on StepOnePlus Real-

Rime PCR System (Applied Biosystems). Amplification were performed under the following

conditions: 95°C for 10 min, 40 cycles of 95°C for 40 s, 59°C for 40 s and 72°C for 40 s and a

melt curve of 95ºC for 15 s, 60ºC for 1 min and 95ºC for 15 s.

Relative quantification of gene expression (qPCR) was performed using the comparative C_T

method (ΔΔCt). Data were obtained from three independent experiments run in triplicate; data

analysis was conducted in Excel and analysis of variance (ANOVA) was carried out. The results

are reported as the means with standard errors (SE). *P* values <0.05 were considered significant.

2.6 *Prediction of putative DNA binding sites for transcriptional factors*

The consensus DNA-binding sequences TATA box, initiator element (INR), downstream

promoter element (DPE), TFIIB recognition element (BRE) and putative transcriptional start site

(TSS) were located using YAPP Eukaryotic Core Promoter program

(http://www.bioinformatics.org/yapp/cgi-bin/yapp.cgi). The CCAAT binding complex box [47],

proximal DNA-binding elements for the carbon catabolite repressor CreA (SYGGRG) [48], and

proximal DNA-binding elements for transcriptional activators controlling genes encoding

xylanolytic and cellulolytic enzymes, Xlnr (GGCTAR) [49, 50], ACE I (AGGCA) [51], ACE II

(GGSTAA) [52, 53], Pac C (GCCARG) [54], AreA (GATA, TATC, GATT) [25, 26, 55] and

Gal4 (CCGTT) [56] were located in *abfA* genes from *Colletotrichum* sp. (Table 1).

2.7 *Phylogenetic analyses*

Phylogenetic analyses were performed on the amino acid sequence deduced for *C.*

lindemuthianum ABFA (647 aa) and 18 α-L-arabinofuranosidase sequences from *Colletotrichum*

sp. genomes. A sequence from the basidiomycete *Pleorotus ostreatus* Type Florida was included

as outgroup (Table 1). Protein sequences were aligned using MUSCLE with MEGA v6.06 [57]. Prior to phylogenetic analyses, peptide signal sequences and N- and C-terminal extensions were excluded. Phylogenetic analysis was performed with the Bayesian Inference (BI), Maximum Likelihood criteria (ML) and Maximum Parsimony in MrBayes Vs. 3.2.2 [58], RAxML (Randomized Axelerated Maximum Likelihood) version 8 [59] and Mega v6.06 [57]. Branch support values were estimated via a bootstrap analysis of 500 replicates and by posterior probabilities (PP). MrBayes runs were performed using the following parameters: amino WAG evolution model with gamma correction, four independent runs of four chains each (one cold chain and three hot chains) for one million generations with sampling every 1000 generations. Trees and parameters were summarized after discarding (burn-in) 25% of the data. The remaining trees were summarized as a majority consensus tree. Trees were visualized using FigTree v1.4.0 [60].

5.   Results and discussion

3.1 *Isolation and sequence analysis of abfA*

DNA encoding α-L-arabinofuranosidase was isolated from each race of *C. lindemuthianum* and the sequences were deposited in GenBank (Accessions: XXXXX, XXXXX). The *abfA* gene of both races is 2231 pb in length with a 5´ and 3´UTR of 20 and 30 bp respectively (Fig. 1). Augustus (gene prediction) analysis showed that *abf*A contains five exons separated by four introns. At the nucleotide and amino acid levels, DNA sequences from both races showed 100% identity. The prediction of *abfA* coding sequence showed an ORF of 1995 bp in length.

Figure text (nucleotide and deduced amino acid sequence):

```
UTR 5'
tagglagacaggacgcaaagATGGTATCATTCAAATCGGCCGTGCTGGCCGCTACATACGTCGTCTCGGCTGCTACTGTCGACATCTCTGTCAAAGCCACCGGAGGCAACGC
                      M  V  S  F  K  S  A  V  L  A  A  T  Y  V  V  S  A  A  T  V  D  I  S  V  K  A  T  G  G  N  A
TACCAGCGGTCATCAATATGGCTTCCTCCACGAGgtcagtccccgtgttccatctgcctctagtaacagactctcacatcagtccagGACATCAACAACTCTGGCGACGGTG
  T  S  G  H  Q  Y  G  F  L  H  E                                                        D  I  N  N  S  G  D  G
GCATCTACGCCGAACTCATCCGCAACCGAGCGTTCCAATACAGCGACAGGTTCCCCGTCTCCCTCGACGGCTGGCACTCCATCAACGGCGCCGTCCTGACCCTCAAGAACCT
  G  I  Y  A  E  L  I  R  N  R  A  F  Q  Y  S  D  R  F  P  V  S  L  D  G  W  H  S  I  N  G  A  V  L  T  L  K  N  L
CGGCGAGCCCCTCTCCGCCGCCCTTCCGGTTCCGTCAACGTTGCTGCTCGCAACGCCACCGGCGCCATCGGTCTCGAGAACGATGGCTACTGGGGCATGGACGTCAGCCAG
  G  E  P  L  S  A  A  L  P  V  S  V  N  V  A  A  R  N  A  T  G  A  I  G  L  E  N  D  G  Y  W  G  M  D  V  R  Q
CAGAAGTACACCGGCTCTTTCTGGGTCAAGGGTGCCTACGAGGGTGTCTTTACGGCCTCCCTCAAGTCTGCGGCTGAACGACGACGTCTTCGGCTCGGTCGAGATCGAGTCCA
  Q  K  Y  T  G  S  F  W  V  K  G  A  Y  E  G  V  F  T  A  S  L  K  S  A  L  N  D  D  V  P  G  S  V  E  I  E  S
GGGCTACCCCCGACGAGTGGGTTGAACACGAGGTTGAACTCGTTCCCACCAAAGACGCGCCCAACAGCAACAACACTTTTGCCATCACCTTTGATTCAGCCgtacgcgcgca
  R  A  T  P  D  E  W  V  E  H  E  V  E  L  V  P  T  K  D  A  P  N  S  N  N  T  P  A  I  T  P  D  S  A
agaaacacgttgccttacgcaacgaaatcgtactgacgtggttggactcagGGCGCTGCCGACGGTTCGCTGGACTTTAACCTCATCAGCCTGTTCCCTCCCACCTATAAGA
                                                     G  A  A  D  G  S  L  D  F  N  L  I  S  L  F  P  P  T  Y  K
ACCGCAAGAATGGCCTGCGCGTCGACATTGCCGAAGCCCTGGCCGGGCTGCACCCCgtaagcagcgactgatcccagagcattacatagcttgctgactatcgaatgcgcag
  N  R  K  N  G  L  R  V  D  I  A  E  A  L  A  G  L  H  P
AGCCTGCTTCGCTTCCCCGGCGGCAACATGCTCGAGGGTATCGACAACTCCACCTACTGGACTGGAAGGACACTCTCGGTCCCCTCAAGGACCGTCCTGGCTTCCAGGGCG
  S  L  L  R  F  P  G  G  N  M  L  E  G  I  D  N  S  T  Y  W  D  W  K  D  T  L  G  P  L  K  D  R  P  G  F  Q  G
TCTGGAACTACCAGCAGACTCACGGACTGGGTCTCGTTGAATACCTGGACTGGGCGGCAGACATGAACCTCAACATTggtaagctcaagctggagcccccatgactccgtaac
  V  W  N  Y  Q  Q  T  H  G  L  G  L  V  E  Y  L  D  W  A  A  D  M  N  L  N  I
tcgtgttgatagatattcgacacgaacaGTCATCGGAGTCTGGGCCGGTCTCGCTCTGAACGGCGACGTCACCCCCAAGGAAGACCTCCAACCGTTCATCGACGACGCCCTCG
                             V  I  G  V  W  A  G  L  A  L  N  G  D  V  T  P  K  E  D  L  Q  P  F  I  D  D  A  L
ACCAGATCGAGTTCATCCGCGGCCCCGTCGACTCGAAATGGGGCGCTCGCCGTGCTGAACTGGGCCACCCCGGAACCCTTCACGCTCGAGTACGTCGAGATCGGCAACGAGGA
  D  Q  I  E  F  I  R  G  P  V  D  S  K  W  G  A  R  R  A  E  L  G  H  P  E  P  F  T  L  E  Y  V  E  I  G  N [E] D
CTGGCTCGCCGGCTACCCCCTGGCTGGACCTCGTACAAGGAGTACGCTTCCCCATGTTCATGAAGGCCATCCGCGCAAAGTACCCAGACATCACCGTCATCTCTTCGGGC
  W  L  A  G  Y  P  L  G  W  T  S  Y  K  E  Y  R  F  P  M  F  M  K  A  I  R  A  K  Y  P  D  I  T  V  I  S  S  G
GCCACCACCGACGGCGACGGCTTCGACATCCCTGCCCCTGGCATCGGCGACTACCACCCTACCGCACCCGGACGCCCTCGTCGACGAGTTCGACCGCTTCGACAACGACG
  A  T  T  D  G  D  G  F  D  I  P  A  P  G  I  G  D  Y  H  P  Y  R  T  P  D  A  L  V  D  E  F  D  R  F  D  N  D
TGGCCCACATCGTCGGCGAGGTCGCGGCGCAGCCCAACGGAGGCACCGGCTGGGACGGCGACCTGATGCCCTTCCCCTGGTGGATCGGCACCGTCGGCGAGGGCGCTC
  V  A  H  I  V  G [E] V  A  A  T  H  P  N  G  G  T  G  W  D  G  D  L  M  P  F  P  W  W  I  G  T  V  G  E  A  V  S
GCTCATCGGCTACGAGCGCAACGCGGACCGCATCCCGGGGACCTTTTACGCCCCCGTGCTGCGCAACATGAACCGCTGGCAGTGGGCCGTCACCATTGTGCAGTTCGCCGCC
  L  I  G  Y  E  R  N  A  D  R  I  P  G  T  F  Y  A  P  V  L  R  N  M  N  R  W  Q  H  A  V  T  I  V  Q  F  A  A
GACCCCGGGCTGACGACGCGCTCGACCAGCTGGTTCGTGTGGGAGCTCTTCGCCGGGCCCACCCCGTGTCCCACACGCTGCCCACCACCGGGGAGTTCGGGACCCGTGTACTACGG
  D  F  G  L  T  T  R  S  T  S  W  F  V  W  E  L  F  A  G  H  P  V  S  H  T  L  P  T  T  G  E  F  G  P  V  Y  Y
TTGCCGGCAAGAACGAGGCCAGGGGCAGCTTCGTCTGGAAGGGTGCTGCGTACAACACGACCACGGCGAGGACGTTCCTGTTCGTCGTCGAGTTTGAGGGTGTCGAGGCCGG
  V  A  G  K  N  E  A  R  G  S  F  V  W  K  G  A  A  Y  N  T  T  D  G  E  D  V  P  V  V  V  E  F  E  G  V  E  A  G
TACCGGAGGCCGCCTTGACTGTTCTCACGAACCCTGGCGGCGACCCGTTGCGTACAACGACCCTCACACCGGTGTCAACATTGTCGACAGCCAGACAGCGTTGTTCTGACTGCG
  T  E  A  A  L  T  V  L  T  N  P  G  G  D  P  F  A  Y  N  I  V  D  S  Q  T  V  V  L  T  A
GACGAGAAGGGTGCTTTCACCTTTGCCCTCCCCGAGCTCAGCGTTGCTGTGTTGGAGACGGATGTAGGTGTTGAGGGTGGCAACAAGACAGCTCGTGGCTGGTCCAAGCCTT
  D  E  K  G  A  F  T  F  A  L  P  E  L  S  V  A  V  L  E  T  D  V  G  V  E  G  G  N  K  T  A  R  G  W  S  K  P
TTTACGGGTAGgctggtgctccatagtcgagcaaaacggtt
  F  Y  A  *
                       UTR 3'
```

Fig. 1. Nucleotide sequence of the *abfA* gene and the deduced amino acid sequence. The underlined amino acid sequence denotes a fragment of the signal peptide. Boxed glutamic acids are putative catalytic residues found in the GH51 family.

Comparison at the amino acid level with corresponding sequences in GenBank revealed a 98% to 83% identity with α-L-arabinofuranosidase genes in other *Colletotrichum* species, 99% identity with *C. lindemuthianum* race 89 *abfA* sequence and a 38% identity with the *A. kawachii* α-L-arabinofuranosiodase (Table 1). In case of *Colletotrichum* species whose genome has no annotation (*C. lindemuthianum* race 89, *C. acutatum* strain 1 KC05_12 and *C. godetiae* C184), we located the genomic sequence of *abfA* genes and made the prediction of coded sequence with

Augustus software [36]. Similarly to *abfA* gene from *C. lindemuthianum* race 0 and 1472, the gene identified in *C. lindemuthianum* race 89 genome, has five exons and four introns. In contrast, the number of introns and exons located in *abfA* genes from other *Colletotrichum* species is variable. There are cases like *C. orbiculare* MAFF 240422 gene, where six exones separated by five introns were localized; *C. salicis* CBS 607.94 gene contains three exons and two introns. In contrast, *abfA* gene from *C. gloesporioides* (strains Nara gc5 and Cg-14) carries only two exons interrupted by one intron. On the other hand, three to four exons were localized in other *abfA* genes of *Colletotrichum* species shown in Table 1. The variable number of exons and introns in *abfA* genes is consistent with genomic *abfA* sequences reported from fungi. *A. kawachii* *AkabfA* gene contains eight exons interrupted with seven introns [24]; in contrast, the *poabf* gene from *P. ostreatus* type Florida has 26 exones separated by 25 introns [27].

Deduced amino acid sequence from *abfa* gene is 665 amino acids in length. According to the SignalP 4.1 web server [39], the mature protein possesses a signal peptide cleavage site between Ala^{17} and Ala^{18}; this finding is consistent with the previously reported *A. kawachii* sequence [24]. According to the ExPASy Proteomics Server [40], the putative mature protein (residues 18 to 665) has a calculated molecular mass of 70607.46 Da and a pI of 4.51. Six putative *N*-glycosylation sites were predicted by the NetNGlyc 1.0 Server at Asp^{30}, Asp^{106}, Asp^{186}, Asp^{251}, Asp^{569} and Asp^{653}; these *N*-glycosylation sites are different from the potential sites reported for AkAbfA from *A. kawachii*, only Asp^{30} coincides with first site proposed to AkAbfA [24]. Several *N*-glycosylation putative sites have been reported for ABFsA from other fungi, AFQ1 from *P. chrysogenum* has seven sites; on the other hand, proteins from *A. niger* presents 10 putative *N*-glycosylation sites [21-23]. NetOGlyc 4.0 Server was used to search *O*-glycosylation sites. One positive site at Thr^{449} was found in ABFA from *C. lindemuthianum*, *O*-glycosylation has been reported to PoAbf from *P. ostreatus*. Mutation analysis at Ser^{160} was performed, demonstrating a

decrease in thermoresistance and pH resistance in mutant arabinofuranosidase, due to an increase in the unfolded structure and decrease in the β-sheet structure content, indicating a role of O-glycosylation in secondary structure stability [61].

Multiple sequence alignment of the amino acid sequence deduced for *C. lindemuthianum* ABF and amino acid sequences for α-L-arabinofuranosidases from *Colletotrichum* species and *P. ostreatus* revealed conserved motifs (Fig. 2). The glutamic acid residues E^{371} and E^{449} found in the *P. ostreatus* PoAbf sequence correspond to acid/base and nucleophile catalytic residues respectively [62]. These residues are conserved in all ABFA sequences of *Colletotrichum* sp.

3.2. *Protein homology modeling*

Homology modeling prediction of the 3D structure of ABFA was performed with I-TASSER software, using a hierarchical method for protein structure prediction. Structures of inactive mutant arabinofuranosidase from *Thermobacillus xylanolyticus* (PDB: 2VRQ), ARAF51 from *Clostridium thermocellum* (PDB: 2C8N, 2C7F) and alpha-L-arabinofuranosidase from *Geobacillus stearothermophilus* (PDB: 1QW9) were used as templates in I-TASSER. Five 3D models were generated. The ABFA model with the most structural similarity to the templates had the following quality values: C-score of -2.73, TM-score of 0.40± 0.13 and a RMSD of 14.7±3.6 Å (Fig. 3). After submit 3D model to ModRefiner software, values of TM-score and RMSD changed to 0.8722 and 10.474 respectively.

Fig. 2. Alignment of α-L-arabinofuranosidase amino acid sequences from *Colletotrichum* species. Percent of conservation is represented with bar plots. Dashes represent gaps introduced to preserve alignment. Underlined residues correspond to the peptide signal. Conserved catalytic residues are indicated with black vertical arrows.
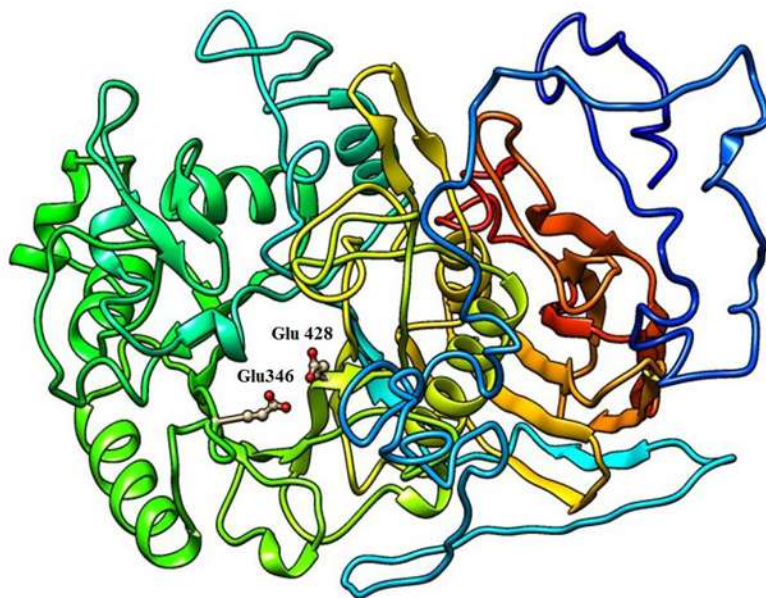
Fig. 3. Protein homology modeling. A 3D image of the tertiary structure of ABFA from *C. lindemuthianum* generated by the I-TASSER server showing the two domains characteristic of GH51 enzymes (catalytic domain with $(\beta/\alpha)_8$ barrel fold and a C-terminal domain with a jelly roll topology). The catalytic amino acids E^{346}, E^{428} are located on the enzyme surface.

Energy minimization of the protein model yielded a value of -11703.267 kJ/mol. A plot of dihedrals allowed the quality of the generated model to be assessed. The results were in agreement with the requirements for preferred and allowed regions, with the exception of 21 (3.2%) non-glycine residues. This model represents the first 3D model of a GH51 α-L-arabinofuranosidase from ascomycete fungi.

Crystallized GH51 ABFA from bacteria presents a multimeric structure composed of a homohexamer with a D_3 point symmetry [63], however, there are no reports of multimeric proteins of GH51 ABFA from fungi. The generated model exhibited a characteristic structure of a monomer of ABFA in bacteria, which is organized in two domains: a catalytic $(\beta/\alpha)_8$ TIM-barrel

fold, and a 12-stranded β-sandwich with a jelly-roll topology at C-terminal [64]. The canonical $(\beta/\alpha)_8$-barrel consists of a closed eight-stranded parallel β-strand, forming a central barrel surrounded by eight α-helices [65]. However, TIM-barrel from ABFA showed some variations, additional subdomain composed of three α-helices and one anti-parallel β-sheet, is inserted between β-strand 2 and α-helix 2. Another α-helix is present between α-helix 3 and β-strand 4 and a short α-helix is inserted between β-strand 7 and α-helix 7. Furthermore, an antiparallel β-sheet is found between β-strand 8 and α-helix 8 [64]. Active-site residues E^{346} and E^{428} are located on the catalytic face of the barrel (Fig. 3).

To compare the 3D ABFA model with arabinofuranosidases A in the GH51 family, a structural alignment between ABFA and the inactive mutant of arabinofuranofuranosidase from *T. xylanolyticus* (PDB: 2VRQ), one of the proteins used as a template in I-TASSER, was performed using the SuperPose Version 1.0 webserver (http://wishart.biology.ualberta.ca/superpose/) [66]. This analysis revealed that both enzymes presents similar topology; however, the enzyme structures do not superimpose completely, ABFA from *C. lindemuthianum* have a longer sequence and an additional loop with a small β-strand located at β-sandwich domain (Fig 4A); likely due to differences in activity and ligand affinity that affect protein folding. ABFA sequence showed 18% of identity and 21% of similarity with ABFA from *T. xylanolyticus*. A comparison of the predicted secondary structures for proteins used by I-TASSER as template and ABFA from *C. lindemuthianum* was performed using PROMALS3D software. This analysis revealed some regions with similar α-helix/β-strand pattern in proteins (Fig 4B), nevertheless, folding of these enzymes present similar topology and active residues located at the same position with small different orientation (Fig 4A). Using the online service Pfam (http://pfam.xfam.org/) [42], an Alpha-L-AF C (Alpha-L-

arabinofuranosidase C-terminal) domain was identified spanning amino acid residues 464 to 639.
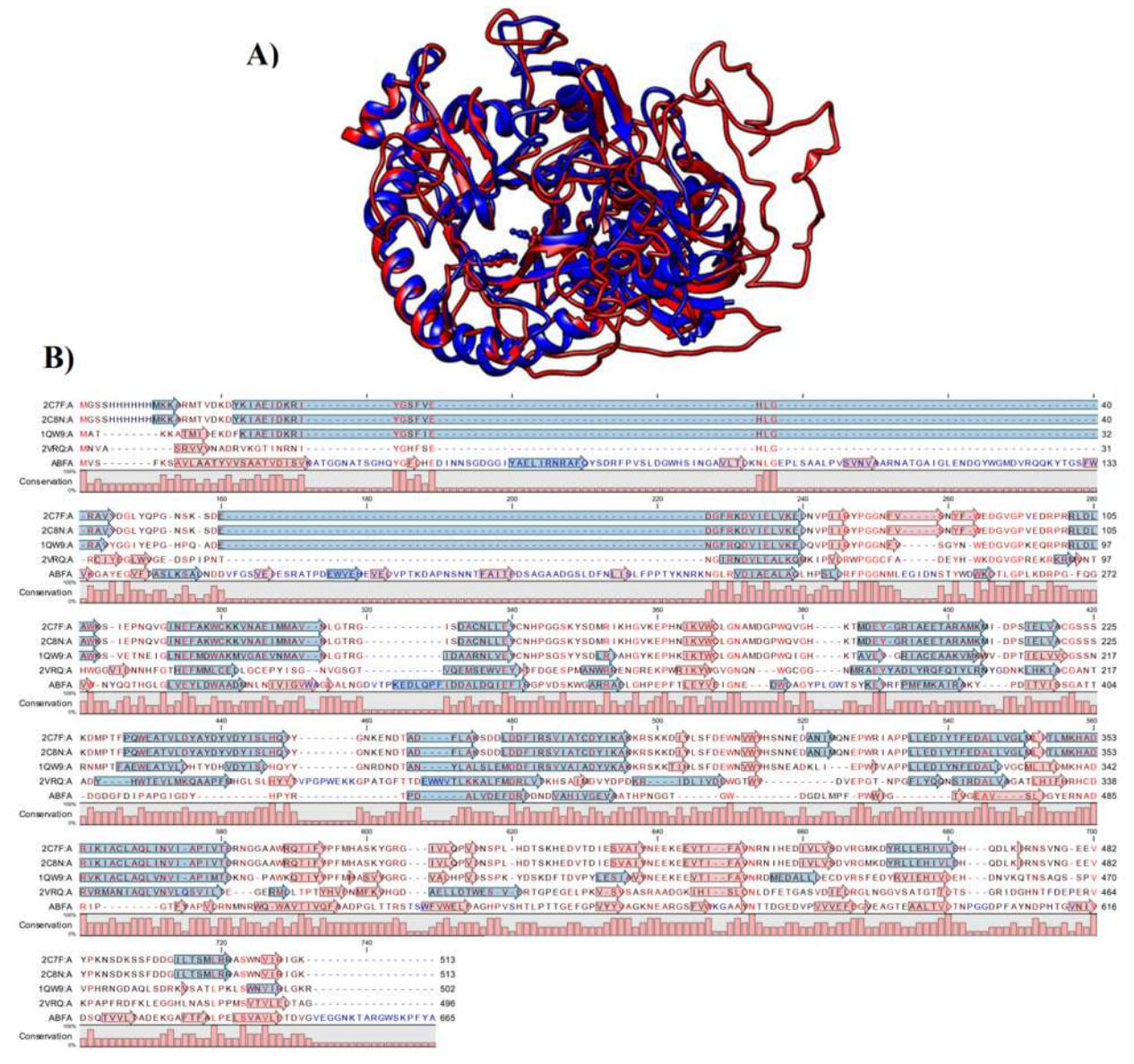

Fig. 4. Superimposition of 3D model structures for inactive mutant of arabinofuranofuranosidase

from *Thermobacillus xylanolyticus* (PDB: 2VRQ) (blue) and ABFA (red) (A). (B), alignment of

ABFA, inactive mutant arabinofuranosidase from *T. xylanolyticus* (PDB: 2VRQ), ARAF51 from

Clostridium thermocellum (PDB: 2C8N, 2C7F) and alpha-L-arabinofuranosidase from

Geobacillus stearothermophilus (PDB: 1QW9) sequences. The secondary structure is showed

with horizontal arrows (α-helix (blue) and β-strands (red)). The bar plots above the sequences indicates conservation.

3.3 *Expression analysis of abfA*

Induction of CW-degrading enzymes by different carbon substrates has been studied in some phytopathogenic fungi such as *Sclerotinia sclerotiorum* [67] *Sclerotium rolsfii* [68] and *Penicillium* sp. [69], among others. The involvement of some of these enzymes in the development of bean anthracnose by *C. lindemuthianum* race γ was first described by Wijesundera *et al.* [70]. Of the secreted polysaccharidases, pectinases [7-10] and xylanases [11] are thought to play a significant role in pathogenesis. Induction of α-L-arabinofuranosidase A gene by different carbon substrates has been studied in some fungi such as *A. kawachii* [24] and *P. chrysogenum* [28]. However, does no exist any study on the role of α-L-arabinofuranosidase in the establishment of the infection.

Results presented here show a clear difference between the non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* in terms of transcript expression when they are grown with different carbon substrates. When glucose was used as the main carbon source, basal expression levels of the *C. lindemuthianum abfA* transcript were observed in both races, suggesting catabolic repression, similar to previously observed to *xyl1* (encoding a β-xylanase) and *ebg* (encoding endo-β-(1,6)-D-galactanase) genes [31, 32]. This results are consistent with Northern-blot analysis and Semi-quantitative RT-PCR analysis performed in *A. kawachii* and *P. chrysogenum AkAbfA* and *afq1* genes using glucose or sucrose as carbon source [24, 28] (Fig. 5). In the models mainly studied as *Aspergillus* sp. and *Trichoderma* sp. the expression of hemicellulases is principally induced by carbon sources [50, 71-73].
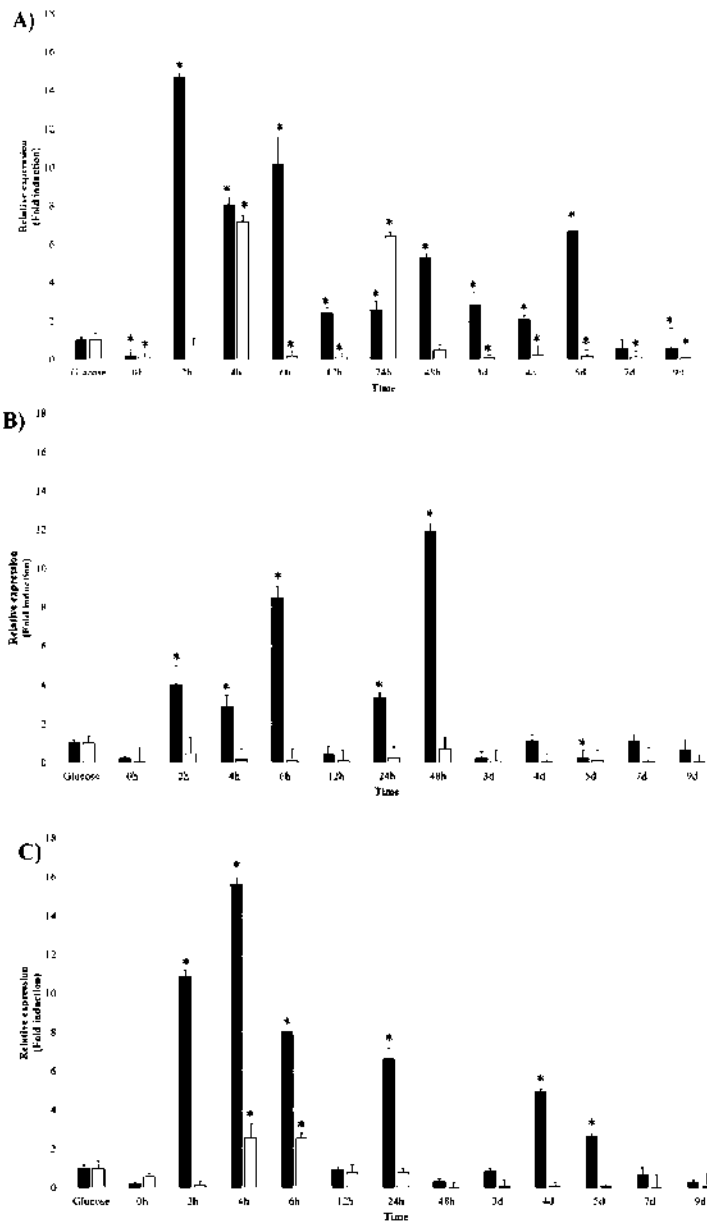
Fig. 5. Analyses of *abfA* expression in pathogenic (1472) and non-pathogenic (0) races of *C. lindemuthianum*. Expression analyses of *abfA* by RT-qPCR of cultures induced with arabinogalactan (a), xylan (b) and bean cell walls (c). Black bars, pathogenic race; white bars, non-pathogenic race. Each bar shows the mean of triplicates ± SE of three independent experiments. The symbol "*" indicates significant changes (*P*<0.05) in relation to the control (glucose).

Transcription is suppressed in the presence of D-glucose, whereas transcription is strongly induced in the absence of D-glucose and presence of hemicellulose [74]. Our results agree with this observation. Thus we used the basal expression with glucose for compare with induction by arabinogalactan, xylan or CW fractions from *P. vulgaris*.

When glucose was replaced with arabinogalactan, *abfA* transcription levels were increased in both fungi. However, the expression profiles differed between the pathogenic and non-pathogenic races: the pathogenic race exhibited three main peaks of *abfA* expression after 2, 6 and 5 days of growth that were respectively 14.67-, 10.17- and 6.64-fold higher than that observed in glucose. In contrast, the non-pathogenic race exhibited two expression peaks 7.36- and 6.38-fold higher than that observed in glucose after 4 and 24 h of growth, but in other times of growth, transcript levels are lower than glucose (Fig. 5a).

It has been reported that a synergistic effect of α-L-arabinofuranosidase, exo-β-(1,3)-D-galactanase and endo-β-(1,6)-D-galactanase is required for arabinogalactan complete degradation [18, 19]. Analysis of endo-β-(1,6)-D-galactanase (*ebg*) gene expression by RT-qPCR performed in mycelium of *C. lindemuthianum* race 1472 growth in arabinogalactan showed higher levels of transcription at 2 and 6 h similar than *abfA* gene. On the other hand, expression of *ebg* in *C. lindemuthianum* race 0 showed a single peak of transcription at 2 h of growth [32]. These results indicate a coordinated gene expression of *ebg* and *abfA* for arabinogalactan degradation.

In the presence of xylan, the pathogenic race exhibited two high peak of *abfA* expression after 6 and 48 h of growth (8.48- and 11.87-fold higher with respect to glucose). Expression of *abfA* declined drastically after 12 h (0.39-fold higher). Thereafter, expression increased over the following 24 and 48 h, reaching a maximum after 48 h (11.87-fold higher) and decreased drastically (0.22 to 1.22-fold higher) in the following 3-9 days of incubation (Fig. 5b). In

contrast, race 0 exhibited low transcript levels, minor or similar to glucose basal expression in all times of growth evaluated (Fig. 5b). Levels of transcript of non-pathogenic race of *C. lindemuthianum* are agree with expression levels reported to *afq1* of the saprophytic fungus *P. chrysogenum,* where constitutive expression at a low level in presence of D-glucose, D-xylose, D-xylitol, D-galactose, L-rhamnose, D-galacturonic acid and slightly induction with L-arabinose, L-arabinitol and arabinoxylan of *afq1*were reported [28]. Levels of transcription of an endo-β-1,4-xylanase (*xyl1*) gene assessed by RT-qPCR in *C. lindemuthianum* race 1472 growth in xylan, were higher after 48 h of induction [31], while higher levels of transcript of *abfA* were detected during first 48 h of induction, suggesting that *xyl1* and *abfA* have a fast and coordinated genetic expression for arabinoxylan degradation.

In *A. niger*, positive regulators XlnR and AraR are involved in the pentose catabolic pathway of L-arabinose and D-xylose. AraR is able to compensate for loss of XlnR on D-xylose, whereas XlnR is hardly able to compensate for loss of AraR on L-arabinose. Our results suggest that xylan degradation products functions as regulators of *abfA* expression in *C. lindemuthianum* pathogenic race 1472, but no in non-pathogenic race 0.

The ability of CW fractions from *P. vulgaris* to induce *abfA* expression in *C. lindemuthianum* was also tested. As observed with arabinogalactan and xylan, higher transcript peaks were detected at first 48 h to pathogenic race; the maximum level of expression was detected at 4h (15.59-fold) with a decrease at 12 h (0.94-fold), similar that occurs in mycelium growth with xylan. After 48 h, a slightly increase of expression levels were observed at four and five days (4.93- and 2.62-fold respectively). Transcript levels in the non-pathogenic race were higher at 4 and 6 h (2.59- and 2.55- fold) decreasing since 12 h reaching values below basal transcription (Fig. 5c). The *abfA* expression profile observed in the pathogenic race over the first 48 h of

growth in the presence of inducing carbon substrates agrees with the pattern of gene expression reported for other hydrolytic enzymes, such as pectin lyase 2 (*Clpnl2*) and *ebg* from *C. lindemuthianum* [30, 32]. Our results indicate that arabinogalactan and xylan degradation products and CW components can induce high levels of *abfA* expression in the pathogenic race and slightly high levels in the non-pathogenic race. In fungi, the expression of extracellular hydrolytic enzymes is coordinately regulated by transcriptional activators and repressors [75]. The expression of genes encoding xylanolytic enzymes is subject to catabolic repression through the action of CreA under a preferred carbon source and the activation through the action of XlnR under carbon limitation [76-78]. The transcriptional activator XlnR regulates genes encoding xylanolytic and cellulolytic enzymes and the use of D-xylose and L-arabinose via pentose catabolic pathway [79, 80]. XlnR homologs are found in the genomes of most filamentous ascomycetes, and they are assumed to have the same function [79, 80].

To determine if in *abfA* genes from *Colletotrichum* genus exists putative DNA-binding sites for transcriptional activators and repressors of CW-degrading enzymes, we performed an analysis for prediction of putative DNA-binding sites in 16 *abfA* genes of 15 species from *Colletotrichum* (Table 1). We did not included analysis of the regulatory region of *abfA* from *C. lindemuthianum*, because not count with its sequences. Instead, we identify and included the regulatory region of *abfA* in the genome reported for *C. lindemuthianum* 89. Initially, the consensus DNA-binding sequences TATA box, initiator element (INR), downstream promoter element (DPE) and putative transcriptional start site (TSS) were located (Fig. 6, Table S1). Thus, the sequence corresponding to the UTR 5´ between the ATG and putative TSS vary between 55 to 1495 bp. Only *C. graminicola* M1.001, *C. chlorophyty* and *C. godetiae* C184 *abfA* promoters have TATA box or TATA-like element, the rest of the sequences have TATA-less core promoter. The upstream transcription factor B-responsive element (BRE) is a proximal element of TATA-box and is

required to initiate transcription by the transcription factor B (TFB) instead of TATA-binding protein (TBP) in arabinose-responsive genes involved in carbohydrate metabolism (Nan et al. 2009). The BRE element was located in five promoters, the DPE element was found in 14 genes, and the INR element was found in 13 genes TATA-less or TATA-like (Table S1). The CCAAT binding complex box (Bc) identified in the promoters of many cellulase and hemicellulase genes and necessary for the full transcriptional activation of certain promoters [47]. Bc binding sites were located in 11 genes except for *C. lindemuthianum* race 89, *C. orbiculare* MAFF 240422, *C. fioriniae* PJ7, *C. higginsianum* IMI 349063 and *C. chlorophyti* (Fig. 6, Table S1). The analysis for prediction of DNA-binding sites it allowed the identification of proximal elements for Cre, Xlnr, ACEI, PacC, AreA and Gal4 transcriptional factors that regulate the expression of genes coding xylanolytic and cellulolytic enzymes (Fig. 6, Table S1). It has been reported that there are two closely Cre binding sites in genes that suffer catabolic repression [75], however, we identify at least two putative sequences in *abfA* from *C. fioriniae* PJ7 and up to 16 putative binding sites in *abfA* from *C. tofieldiae* CBS 130852 and *C. chlorophyti*. Xlnr binding sites were identified only in eight genes. ACEI is a negative regulator of cellulases and xylanases in *T. reesei* [81]. ACEI binding sites (1 to 5) were found in most *abfA* genes but were absent in *C. gloeosporioides* Cg-14, *C. gloeosporioides* Nara gc5, *C. graminicola* M1.001 and *C. orchidophilum* IMI 309357. It was reported that ACEII is an essential universal activator controlling the transcription of cellulases and hemicellulases [82]. However, we did not find ACEII binding sites in any gene.

PacC is an activator for alkaline-expressed genes [75], PacC binding sites (1 to 3) were found in 11 genes. The binding sites for Gal4, a transcriptional factor that regulates the galactose catabolism in yeast [78] were located in *C. lindemuthianum* race 89, *C. gloeosporioides* Cg-14, *C. tofieldiae* CBS 13085, *C. salicis* CBS 607.94 and *C. incanum* MAFF 238704.

AreA, also knows as GATA factor, is a regulator involved directly in chromatin remodeling [83], it has been demonstrates that this factor is implicated in cellulases activity [55]. All of analyzed promoters of *abfA* have a putative binding site (2-26) for AreA (Fig. 6). Our results are agree with analysis of putative transcriptional regulator binding sites in the promoter region of family 51 fungal ABF genes showed several binding sites to CreA (1-12), 1-2 sites to PacC, 0-1 sites to XnlR, 1-7 sites to AreA and at least one binding site to ACEI [26].

These results support the idea that *abfA* genes from *Colletotrichum* species are under regulation of transcriptional factors that regulate the expression of genes xylanolytic and cellulolytic enzymes. However, is necessary the development of studies that confirm the function of the detected DNA consensus sequences and proximal elements. The question of how the *abfA* genes are regulated can be answered by this way.
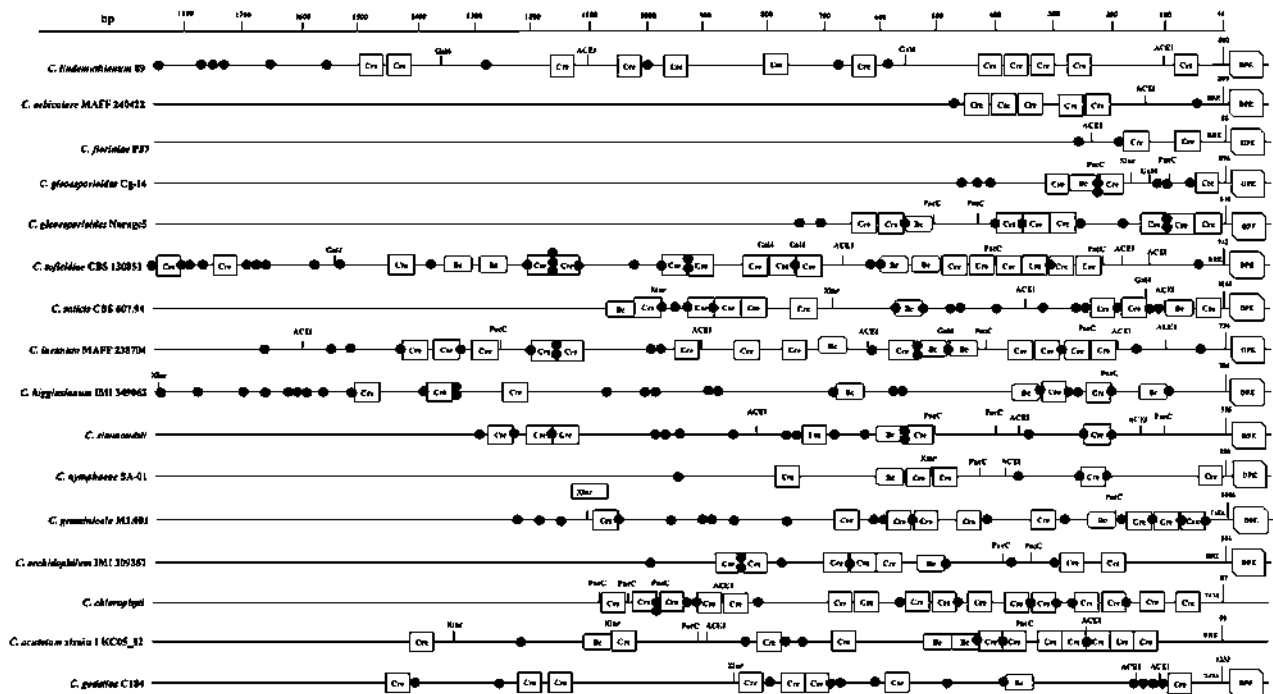
Fig 6. Putative DNA binding sites for transcriptional factors that regulate genes coding CW-degrading enzymes, identified in *abfA* genes. TSS (transcriptional start site) number (+1) indicates the distance with the start codon of each sequence (ATG). Cre (carbon catabolite repressor), Bc (CCAAT binding complex box), DPE (downstream promoter element), BRE (TFIIB recognition element), black circles (AreA).

Another question is respect to the ABFs function beyond the generation of a carbon source for the fungus. Previously, we proposed that different *ebg* expression profiles between pathogenic and non-pathogenic races of *C. lindemuthianum* plays a role in the degradation of plant CW to establish the infection [32]. The endo-β-(1-6)-D-galactanase (EC 3.2.1.164) is a debranching enzyme, catalize the hydrolysis of β-(1-6)-galactosyl side chains of arabynogalactans (AGs) and produce β-(1-6)-galacto-oligomers and β-(1-6)-galactobiose [84, 85] but EBGs only act on desarabynosilated substrates [85, 86], therefore an ABF activity is essential to EBG action.

Since the carbohydrate groups in AGPs are critical for their function [87], it is conceivable to hypothesize that the pathogenic race of *C. lindemuthianum*, which more rapidly expresses *ebg* and *abfA* at higher levels in the presence of plant CW polysaccharides, is better adapted to degrade AGPs for the establishment of the infection.

3.4 *Phylogenetic analyses*

To elucidate the relationship between ABFs A from *C. lindemuthianum* race 0, 89 and 1472 and ABFs A from other *Colletotrichum* species, the amino acid sequence of *C. lindemuthianum* ABFA and 18 sequences for the same gene from 16 species were analyzed phylogenetically. The ABFA sequence of the basidiomycete *Pleorotus ostreatus* Type Florida was used as outgroup

(Table 1). The results showed that ABFs A are orthologs proteins separated into two clades (Fig.

7). In clade I the arabinofuranosidase from *C. lindemuthianum* race 0 and 1472 was grouped with

the ABFs A from *C. lindemuthianum* race 89 and *C. orbiculare* MAFF 240422, the nearest

species according to a phylogenetic analysis of *Colletotrichum* sp. [88, 89]. ABFs A from *C.

gloeosporioides* (Cg-14, Nara gc5) are grouped in the same clade. On the other hand,

Colletotrichum species that infects monocots are also grouped in clade I, *C. sublineola* and C.

graminicola M1.1001 are located in one clade, while ABF of *C. orchidophilum* IMI 309057 is

basal of clade I. Into clade II were grouped *Colletotrichum* species pathogenic of dicots.
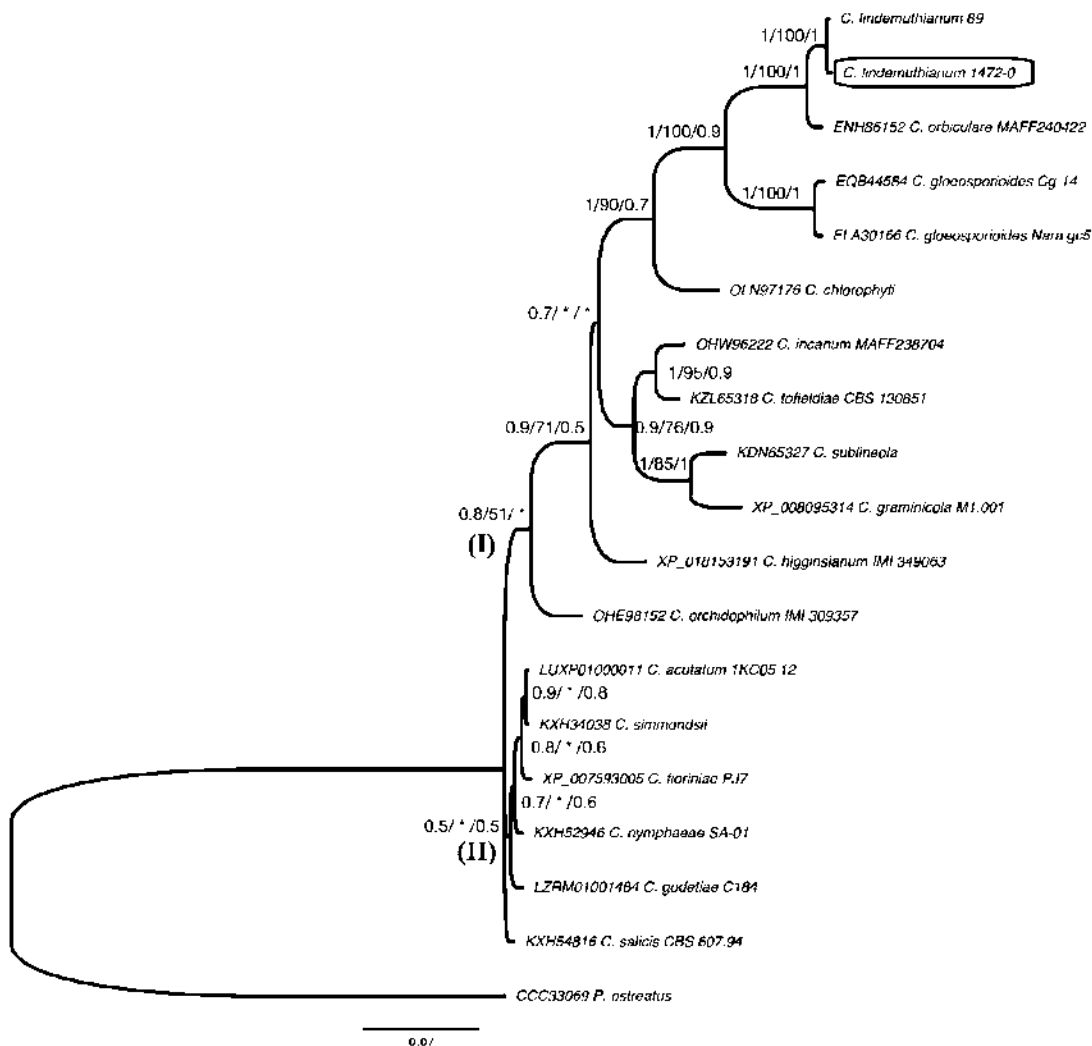
Fig. 7. Phylogenetic analyses of α-L-arabinofuranosidases from *Colletotrichum* species. The phylogenetic tree was constructed using the BI, ML and MP methods. The general topology obtained is represented by the Bayesian 50% majority rule consensus tree. BI posterior probabilities and ML bootstrap support are indicated on the branches. The numbers at the nodes indicate bootstrap values based on 1,000 bootstrap replications.

There have been no previous reports regarding phylogenetic analyses of α-L-arabinofuranosidases A of *Colletotrichum* genus. Our results it does show a diversification of ABFs A into different groups or lineages. It has been observed that pathogenic fungi of monocot and dicot plants are better adapted to degrade the cell walls of monocot and dicot plants, respectively; this phenomenon clearly reflects host preference [90]. Transcriptome analyses of *C. graminicola* and *C. higginsianum* during interactions with monocots and dicots, which exhibit different CW compositions, have revealed differences in the expression levels of polysaccharidases. In the case of *C. higginsianum*, such differences allow the organism to invade a broader range of host plants [91]. In our analysis, although two groups or lineages of ABFs included pathogens of dicot and monocot plants (Clade I), one lineage of ABFs A included pathogens of dicot plants and an entomopathogen (*C. fioriniae* PJ7) (Clade II), suggesting that evolution of some ABFs A is not necessarily influenced by the host preference.

6. Conclusion

This study is the first report of cDNA isolation, 3D protein modeling, phylogenetic and expression analyses of α-L-arabinofuranosidase A a GH51 family protein, from *C. lindemuthianum*. Our results revealed different *abfA* expression profiles between pathogenic and

non-pathogenic races of *C. lindemuthianum,* suggesting that this enzyme plays a role in the degradation of plant CW to establish the infection. Besides the determination of the putative consensus DNA binding sequences TATA box, INR, DPE and TSS in 16 *abfA* genes from 15 species of the genus *Colletotrichum*, we found putative DNA binding sites for transcriptional factors Cre, Xlnr, ACEI, PacC, AreA and Gal4 that regulate the expression of genes coding CW-degrading enzymes. Phylogenetic analysis of ABFs A from *Colletotrichum* species shows three protein lineages.

Acknowledgements

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Supplementary material

Table S1. Consensus DNA-binding sequences and putative DNA-binding sites for transcriptional factors located in *abfA* genes from *Colletotrichum* species.

Especie	BRE	TATA	INR	TSS	DPE	BC	CRE	Xnlr	ACE I	ACE II	PacC	Gal4	AreA
C. lindemuthianum 89			CCACTCC (-1/+5)	810	GGATC (+28/+32)		CCGGG (-76/-72) (-1450/-1446) CTGAG (-223/-219) CCGAG (-301/-297) (-369/-365) (-795/-791) GTGAG (-941/-937) (-1136/-1132) CTGGG (-339/-335) (-605/-601) (-1036/-1032) (-1484/-1480)		AGGCA (-101/-97) (-1113/-1109)			CCGTT (-542/-538) (-1345/-1341)	GATA (-593/-590) (-1000/-997) (-1716/-1713) TATC (-1289/-1286) (-1547/-1544) (-1640/-1637) (-1802/-1799) GATT (-660/-657) (-1752/-1749) (-1760/-1757)
C. orbiculare MAFF 240422	CCACGCC (-31/-25)		CCAATAT (-2/+4)	879	AGACG (+28/+32)		GCGGG (-247/-244) (-284/-280) CTGGG (-363/-359) CCGAG (-393/-389) (-402/-398)		AGGCA (-131/)				GATA (-469/-466) TATC (-60/-57)
C. fioriniae PJ7	GGACGCC (-35/-29)		TCAGTTT (-2/+4)	55	AGACG (+28/+32)		GTGGG (-82/-86) CCGGG (-173/-177)		AGGCA (-231/-235)				TATC (-198/-195) (-260/-257)
C. gloeosporioides Cg-14			TCAATTC (-1/+5)	876	GGATG (+28/+32)	CCAAT (-241/-245)	GCGAG (-43/-47) (-215/-219) GCGGG (-275/-279)	GGCTAG (-144/-149)			GCCAAG (-139/-144) (-225/-230)	CCGTT (-158/-162)	GATA (-71/-68) (-250/-247) (-426/-423) TATC (-133/-130) (-248/-245) GATT (-102/-99) (-406/-403) (-471/-468)
C. gloeosporioides Nara gc5			CCACATT (-2/+4)	610	AGTCC (+28/+32)	CCAAT (-525/-529)	CTGAG (-32/-28) CCGGG (-107/-103) (-626/-622) GTGGG (-109/-113) CCGAG (-318/-322) GCGGG (-350/-354) (-385/-389) (-559/-563)				GCCAAG (-419/-414) (514/-509)		GATA (-101/-98) (-180/-177) (-712/-709) TATC (-99/-96) (-408/-405) (-539/-536) GATT (-265/-262) (-377/-374) (-757/-754)
C. tofieldiae CBS 130851	GGGCGCC (-32/-26)			742	AGACC (+28/+32)	CCAAT (-454/-450) (-538/-534) (-1225/-1221) (-1388/-1384)	GCGAG (-312/-308) (-372/-368) (-423/-419) GTGAG (-1163/-1159) (-1762/-1758) (-1873/-1869) GCGGG (-256/-252) (-968/-964) (-1407/-1403) GTGGG (-331/-327) (-930/-926) CTGAG (-411/-407) (-762/-758) (-829/-825) CTGGG (-787/-783) (-1173/-1169)	GGTAA (-2110/-2105)	AGGCA (-122/-118) (-205/-201) (-647/-643)		GCCAAG (-226/-221) (-438/-433)	CCGTT (-749/-745) (-768/-764) (-1558/-1554)	GATA (-61/-58) (-597/-594) (-893/-890) (-934/-931) (-1125/-1122) (-1178/-1175) (-1705/-1702) (-1788/-1785) TATC (-300/-277) (-743/-740) (-993/-900) (-1118/-1115) (-1176/-1173) (-1396/-1393) (-1535/-1532) (-1661/-1658) (-1700/-1697) (-1923/-1920) GATT (-630/-627) (-1040/-1037) (-1150/-1147) (-1611/-1608) (-1781/-1778) (-1869/-1866) (-1944/-1941) (-2010/-2007)
C. salicis CBS 607.94			CTATTTG (-1/+5)	1148	AGACG (+28/+32	CCAAT (-55/-51) (-517/-513) (-1024/-1020)	GCGAG (-960/-956) (-983/-979) GTGAG (-10/-6) (-714/-710) (-1056/-1052) GCGGG (-207/-203) GTGGG (-843/-839) (-909/-905) CCGAG (-170/-166)	GGCTAG (-688/-683) (-991/-986)	AGGCA (-117/-113) (-354/-350)			CCGTT (-149/-145)	GATA (-163/-160) (-461/-458) (-979/-976) TATC (-185/-182) (-256/-253) (-267/-264) (-281/-278) (-332/-329) (-467/-464) (-556/-553) (-913/-910) (-965/-962) GATT (-419/-416) (-572/-569) (-937/-934)
C. incanum MAFF 238704			CCATTCG (0/+7)	774	GGACC (+28/+32)	CCAAT (-426/-422) (-510/-506) (-668/-664)	GCGGG (-233/-229) (-966/-962) (-1397/-1393) GCGAG (-291/-287) (-351/-347) (-1199/-1195) CTGAG (-383/-379) (-1274/-1270) CCGAG (-518/-514) CTGGG (-762/-758) (-805/-801) CCGGG		AGGCA (-102/-98) (-184/-180) (-619/-615) (-911/-907) (-1586/-1582)		GCCAAG (-205/-200) (-410/-405) (-1246/-1241)	CCGTT (-483/-479)	GATA (-42/-39) (-1313/-1310) (-1431/-1428) (-1512/-1509) (-1555/-1552) TATC (-279/-276) (-586/-583) (-951/-948) (-1052/-1049) (-1172/-1169) (-1180/-1177) (-1688/-1685) GATT (-172/-169) (-440/-437)

Species												
							(-1169/-1165) GTGGG (-1374/-1370)					(-534/-531) (-602/-599) (-1146/-1143)
C. higginsianum IMI 349063			CCATTCG (+1/+6)	784	GGACC (+28/+32)	CCAAT (-192/-189) (-347/-343) (-646/-642)	GTGAG (-1214/-1210) GTGGG (-246/-242) (-1371/-1367) CCGAG (-1494/-1490) CTGGG (-304/-300)	GGCTAA (-1831/-1827)		GCCAAG (-272/-267)		GATA (-319/-316) (-377/-374) (-557/-554) (-884/-881) (-1604/-1601) (-1913/-1910) TATC (-215/-212) (-593/-590) (-867/-864) (-1064/-1061) (-1334/-1331) (-1384/-1381) (-1552/-1549) (-1602/-1599) (-1644/-1641) (-1869/-1866) GATT (-107/-104) (-267/-264) (-679/-676) (-932/-929) (-1007/-1004) (-1344/-1341) (-1506/-1503) (-1594/-1591) (-1730/-1727) (-1768/-1765)
C. simmondsii			CCAATCC (-1/+5)	555	AGACG (+28/+32)	CCAAT (-584/-580)	GCGGG (-558/-554) (-704/-700) (-1261/-1257) GTGGG (-1149/-1145) CCGGG (-260/-256) CTGAG (-1153/-1149)		AGGCA (-139/-135) (-399/-395) (-826/-822)	GCCAAG (-531/-526) GCCAGG (-103/-98) (-402/-397)		GATA (-254/-251) (-335/-332) (-617/-614) (-737/-734) (-829/-826) (-952/-949) (-1296/-1293) TATC (-252/-249) (-689/-686) GATT (-541/-538) (-566/-563) (-769/-766) (-931/-928) (-1003/-1000) (-1167/-1164) (-1217/-1214)
C. nymphaeae SA-01			CCAATCC (-1/+5)	555	GGACC (+28/+32)	CAAT (-594/-590)	GCGAG (-13/-9) (-561/-557) CCGGG (-270/-266) GCGGG (-568/-564) (-795/-791)	GGCTAA (-542/-537)	AGGCA (-409/-405)	GCCAGG (-412/		GATA (-264/-261) (-345/-342) TATC (-262/-259) (-932/-929)
C. graminicola M1.001		CCGATAAATCAG (-23/-12)		1495	AGATA (+28/+32)	CCAAT (-231/-235)	GTGGG (-75/-79) (-304/-300) GCGAG (-118/-122) GCGGG (-185/-189) CTGAG (-455/-451) (-557/-553) CCGAG (-592/-596) (-676/-672) CTGGG (-1087/-1083)	GGCTAA (-1068/-1063)		GCCAAG (-216/-211)		GATA (-65/-62) (-425/-422) (-952/-949) (-1184/-1181) TATC (-155/-152) (-195/-192) (-765/-762) (-859/-856) (-917/-914) (-1061/-1058) GATT (-105/-102) (-110/-107) (-279/-276) (-572/-569) (-592/-589) (-623/-620) (-896/-893) (-1164/-1161) (-1212/-1209)
C. orchidophilum IMI 309357	GCGCGCC (-36/-30)		CCAATCC (+2/+8)	544	AGACC (+28/+32)	CCAAT (-530/-526)	CCGGG (-217/-213) (-841/-836) GTGAG (-300/-296) GTGGG (-631/-627) GCGGG (-639/-635) (-825/-821) CCGAG (-788/-784)			GCCAAG (-324/-319) (-395/-390)		GATA (-291/-288) (-1017/-1014) TATC (-381/-378) (-526/-523) (-653/-650) (-865/-862) GATT (-777/-774) (-847/-844)
C.chlorophyti		GATATAAAGCCG (-23/-12)	CCACATC (+2/+8)	87			GCGAG (-422/-418) GTGAG (-132/-128) (-1063/-1059) GCGGG (-58/-54) (-211/-207) (-233/-229) (-526/-522) (-833/-829) (-888/-884) (-1077/-1073) GTGGG (-666/-662) (-1030/1026) CCGGG (-329/-325) (-638/-634) CTGGG (-398/-394) (-536/-532)		AGGCA (-877/-873)	GCCAAG (-958/-953) (-1044/-1040) (-1059/-1055)		GATA (-188/-185) (-278/-275) (-325/-322) (-825/-822) (-932/-929) (-950/-947) TATC (-481/-478) (-992/-989) GATT (-320/-317) (-556/-553) (-995/-992)
C. acutatum STRAIN 1 KC05_12	GGACGCC (-31/-25)		TCAGTTT (+2/+8)	59		CCAAT (-447/-443) (-497/-493) (-1087/-1083)	GCGAG (-205/-201) GCGGG (-242/-238) (-372/-368) (-1061/-1057) (-1404/-1400) CCGAG (-414/-410) CCGGG (-173/-169) (-269/-265) (-763/-759) CTGAG (-253/-249) CTGGG (-672/-668)	GGCTAA (-1035/-1030) (-1331/-1326)	AGGCA (-231/-227) (-902/-898)	GCCAAG (-357/-352) GCCAGG (-905/-900)		GATA (-757/-754) (-838/-835) TATC (-256/-253) (-386/-383) (-420/-417) (-755/-752) GATT (-1219/-1216)
C. godetiae C184		CGGATATATTCT (-24/-13)		1332	AGACA (+28/+32)	CCAAT (-363/-359)	GCGAG (-814/-810) GTGGG (-561/-557) (-1200/-1196) GCGGG (-66/-62)	GGCTAG (-845/-840)	AGGCA (-157/-153) (-202/-198)			GATA (-617/-614) TATC (-115/-112) (-129/-126) (-180/-177) (-207/-204)

						(-1432/-1428) GTGGG (-763/-759) CCGGG (-1174/-1170) CTGAG (-745/-741)					(-397/-394) (-483/-480) (-1418/-1415) GATT (-661/-658) (-721/-718) (-791/-788) (-1258/-1255)

Capítulo 4

Artículo de Investigación que será sometido a la revista BIOTECHNOLOGY LETTERS.

Se reporta la determinación de actividad enzimática de la α-L-arabinofuranosidasa en dos razas de *C. lindemuthianum* con distinto estilo de vida. Adicionalmente, la purificación y caracterización de la misma a partir de la raza 1472 de *C. lindemuthianum*.

Extracellular activity, purification and biochemical characterization of an α-L-arabinofuranosidase from *C. lindemuthianum* race 1472

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Abstract

Objectives: Compare the production of α-L-arabinofuranosidases in pathogenic and non-pathogenic races of *C. lindemuthianum;* purify and biochemical characterization of enzyme.

Results: the pathogenic and non-pathogenic races of *C. lindemuthianum* are able to form and secrete ABF activity when grown under different culture conditions. Xylan was the best inductor of growth and extracellular ABF activity for both races. ABF was purified 91.8 folds with a specific activity of 331.6 nM of 4-MU/min/μg of mycelium protein. Molecular mass of 82 kDa, optimum pH = 6.0, optimum temperature at 50ºC and activation by divalent cations, mainly Ca^{2+}. Apparent K_m and V_{max} values on 4-MU-ABF were 0.23 mM and 303.03 nM of 4MU/min/μg of mycelium protein.

Conclusions: Wide range of pH and temperature, as well as high substrate specificity, which make ABF from *C. lindemuthianum* a good candidate to be used in biotechnology and industrial process.

Introduction

Colletotrichum lindemuthianum (Sacc. and Magnus) Lams-Scrib. is an economically important hemibiotrophic phytopathogen that causes antrachnose disease in common bean (*Phaseolus vulgaris* L.), causing up to 100% yield loss (Padder et al. 2017). *Colletotrichum* genus was classified in eighth place of the 'Top 10' fungal pathogens based on its scientific/economic importance (Dean et al. 2012). Particularly, *C. lindemuthianum* encompasses different strains or special forms known as races, physiological races or pathotypes. These pathotypes are classified based on their interactions with a group of 12 different *P. vulgaris* cultivars (Rodríguez-Guerra et al. 2006). A non-pathogenic race and more than 100 pathotypes with different levels of virulence have been reported around the world. AFLP analyses of 10 of the 54 *C. lindemuthianum* pathotypes identified in México have found high genetic diversity and several lineages (Rodríguez-Guerra et al. 2006; Sánchez-García et al. 2009). During the biotrophic phase of fungal infection, a spore adheres to the plant surface and germinates to form a short germ tube, which differentiates into a melanized appresorium. This structure transforms internal pressure into mechanical force, allowing for penetration of the cuticle and epidermal cell wall (CW) and the formation of a vesicle and a primary hyphae within the cell (Mendgen and Hahn 2002). A necrotrophic phase then follows, which begins 48-72 h after germination. This necrotic phase is characterized by the development of a secondary hyphae, which grows both intra- and intercellularly within plant tissues and secretes large amounts of various polysaccharidases that collectively degrade the host CW (Mendgen and Hahn 2002; Münch et al. 2008). Of the secreted polysaccharidases, pectinases and xylanases are thought to play a major role in pathogenesis (Villa-Rivera et al. 2017). Specifically, production of pectin lyase II, α-arabinofuranosidase, α– and β-galactopyranosidase and protease were detected in lesions of hypocotyls of *P. vulgaris* inoculated with *C. lindemuthianum*. Additionally, endo-polygalacturonase, α-arabinofuranosidase, α- and β-galactopyranosidase and protease were secreted into media with sodium polypectate or cell walls isolated of *P. vulgaris* hypocotyls as main carbon source (Wijesundera et al. 1989). In this sense, production of extracellular cellulolytic enzymes by cellulose induction was realized in *C. lindemuthianum* race 1472 (Acosta-Rodríguez et al. 2005). Additionally, comparison of fungal growth and production of extracellular pectin lyase (PNL) and xylanase (XYL1) activity by pathogenic and non-pathogenic races of *C. lindemuthianum* cultivated under different conditions was evaluated (Conejo-Saucedo et al. 2016; Hernández-Silva et al. 2007). Complete degradation of plant cell walls requires coordinated and synergistic action of principal and accessory enzymes (Conejo-Saucedo et al. 2011)). Among of the most important accessory enzymes secreted by fungi are the α-L-

arabinofuranosidases (ABFs) (EC3.2.1.55). ABFs are hydrolytic cell wall exo type enzymes, which catalyzes the hydrolysis of arabinosyl branches attached through α-L-(1,2), α-L-(1.3), α-L-(1,5) O-glycosidic anchors to arabinoxylans, arabinoxilooligosaccharides, arabinan, arabinogalactans and arabino-oligosaccharides (Lagaert et al. 2014; Numan and Bhosle 2006; Saha 2000). This polysaccharidases are classified in GHs 2,3,10, 43, 51, 54, 62 based in their amino acid sequence according to Carbohydrate-Active enZYmes database (CAZY).

ABFs act synergistically with xilanases and β-xilosidases in arabinoxylan depolymerization (Conejo-Saucedo et al. 2011; Hashimoto and Nakata 2003); on the other hand, a synergistic effect of α-L-arabinofuranosidase, exo-β-(1,3)-D-galactanase and endo-β-(1,6)-D-galactanase is required for arabinogalactans type II complete degradation (Okawa et al. 2013; Sakamoto and Ishimaru 2013). Thus, ABFs have been applied in different agro-industrial processes; production of arabinose as antiglycemic agent, production of antimetastatic and anticarcinogenic compounds, in wine industry, acetic acid production and quality of the bread, improvement of animal feedstock, pulp and paper industry, juice industry, production of fermentable sugars for bioethanol industry and synthesis of pentose-containing compounds (Numan and Bhoshe 2006). Because of the potential applications in biotechnology, native ABFs have been purified and characterized from fungi: *Aspergillus niger* (Flipphi et al. 1994; Rombouts et al. 1988; vd Veen et al. 1991), *A. awamori* (Kaneko et al. 1998), A. *kawachii* (Koseki et al. 2003)*, Penicillium capsulatum* (Filho et al. 1996); *P. chrysogenum (Sakamoto and Kawasaki 2003)*, *P. purpurogenum* (Fritz et al. 2008). Also, recombinant ABFs of *A. oryzae* (Matsumura et al. 2004), *A. niger* (Alias et al. 2011), *Pleorotus ostreatus* (Amore et al. 2012) and *P. chrysogernum* (Sakamoto et al. 2013) have been purified and characterized.

A useful approach to evaluate the biotechnological potential of ABFs and other cell-wall-degrading enzymes produced by fungi would be to compare the production of these enzymes in cultures with complex natural substrates of pathogenic and non-pathogenic races. Here we report the growth and production of extracellular ABF activity by non-pathogenic and pathogenic races of *C. lindemuthianum* cultivated in media containing arabinogalactan, xylan and cell wall from *Phaseolus vulgaris* as carbon sources. Additionally, native ABF of pathogenic race was purified and some biochemical characteristics are reported. This job represents the first report of ABF purified from *Colletotrichum* genus.

Materials and methods

Strains and culture conditions

C. lindemuthianum races 0 (non-pathogenic) and 1472 (pathogenic) were kindly provided by Dr. June Simpson (CINVESTAV-IPN, Unidad Irapuato, Mexico) and maintained on potato dextrose agar (French and Hebert 1980) at 18°C. For enzymatic activity analysis, 1.6 mg (approximately 8 cm^2) of mycelia from both races was inoculated into 125 ml-Erlenmeyer flasks containing 50 ml of modified Mathur's medium (Acosta-Rodríguez et al. 2005) supplemented with 2.5% of glucose, arabinogalactan (from larchwood; Sigma-Aldrich, St. Louis, MO, USA), xylan (from beechwood; Sigma-Aldrich, St. Louis, MO, USA) or CW from *P. vulgaris* (cv. Flor de Mayo) obtained from hypocotyls as described elsewhere (Fry 2006). After different periods of time, cultures were filtered with Whatman filter paper of 3mm in a Nalgene Filter Flasks coupled to a vacuum pump. Cell free extract were kept on ice.

Growth was measured as mg of dry mycelia except where plant cell walls were used as carbon source, in this case, it was measured as the amount of mycelial extracellular protein due to residual plant cell walls interfered with weight quantitation. Subsequently, 5 ml of cell-free medium (CFM) was filtered through a column (1.5 X 10 cm) of Bio-Gel P-6, equilibrated and eluted with 50mM sodium acetate buffer (buffer A), pH 5.0. Fractions corresponding to the void volume (Vo) were pooled; the pool was designated as filtered extracellular medium (FEM) and used to determine protein and ABF activity.

To ABF purification, a pre-inoculum from *C. lindemuthianum* race 1472 was growth in potato dextrose (PD) medium (French and Hebert 1980) and shaken (125 rpm) at 18°C. After 8 days, mycelia were collected by filtration, washed with water and transferred to 500 ml-Erlenmeyer flasks containing 250 ml of modified Mathur's medium (Acosta-Rodríguez et al. 2005) supplemented with 2.5% of arabinogalactan (from larchwood; Sigma-Aldrich, St. Louis, MO, USA). Flasks were shaken (125 rpm) at 18ºC to induce ABF activity. After 6 days of induction, cultures were filtered as described above.

Assay of ABF activity and protein quantitation

Enzyme activity was measured by a fluorogenic method using 4-methylumbelliferyl-α-L-arabinofuranoside (4-MU-ABF) (Sigma-Aldrich St. Louis, MO, USA). Reaction mixtures containing 5 μM 4-MU-ABF, FEM and buffer A in a final volume of 100 μl. The mixtures were incubated during 45 min at 50ºC. The reaction was stopped with 1 ml of 0.5 M Na_2CO_3 - 0.1N NaOH, pH 10.4, liberated 4-MU was measured in a Varioskan Flash (Thermo Scientific) with exitation and emission at 350 and 440 nm respectively. Protein concentration in each sample was determined by

Bradford´s method using Coomassie (Bradford) Protein Assay Kit, according to the manufacturer´s instructions (Thermo Scientific). ABF activity was expressed as specific activity of nM of 4-MU liberated/min/µg protein.

Significant changes in growth and extracellular activity of ABF between pathogenic and non-pathogenic races of *C. lindemuthinaum* analyzed through *t* student test, P values of <0.05 were considered significant.

Enzyme purification

Cell-free medium was concentrated by ultrafiltration using Amicon Ultra Centrifugal Filters (Millipore). To eliminate substrate remnant, concentrated medium was passed through desalting column (HiTrap desalting, 5 ml GE) equilibrated and eluted with 50 mM sodium acetate, pH 7.0. Fractions were pooled and concentrated by ultrafiltration as described above. The enzyme was purified to homogeneity in two purification steps. As first step, anionic exchange chromatograpy was performed using Hi Trap DEAE, 1 ml column (GE), equilibrated with 50 mM sodium acetate, pH 7.0. The sample was eluted from the column with stepped increases in NaCl from 0.3 to 0.8M in 50 mM sodium acetate, pH 7. The active fractions collected were pooled and concentrated by ultrafiltration. Second purification step was performed by gel filtration through HiLoad 16/600 Superdex 75 prep grade (GE) column. The column was equilibrated and eluted with phosphates buffer (0.05 M Na_2HPO_4, 0.05 M NaH_2PO_4) pH 7.2 containing 0.15 M NaCl. Fractions of 1 ml were collected and their enzyme activities were measured as described above.

Electrophoresis and zymogram

SDS-PAGE was performed according to Laemmli (1970) using 10% acrylamide. Protein bands were stained with Coomassie G-250 using SimplyBlue Safe Stain kit (Life Technologies). Zymogram was performed using a 2% low-melting agarose gel, prepared in 50 mM sodium acetate buffer pH 7 containing 50 µg/ml 4-MU-ABF and laid over SDS-PAGE gel. After 30 min incubation at 37ºC, the gel was visualized under UV light.

Enzymatic properties

The effect of pH on the ABF activity was determined using 100 mM sodium acetate buffer with pH values from 3 to 7 and 100 mM Tris-HCl with pH values from 7.5 to 9. The ABF activity was measured by the standard method. The effect of temperature on enzymatic activity was evaluated. Enzymatic reaction mixtures were incubed at 30, 37, 50, 60, 70 and 80ºC. Time of incubation effect was also determinate testing 20, 40, 60 and 80 min and measured by

standard method. To evaluate the possible effect of divalent cations and chelators on enzymatic activity, increasing concentrations of MgCl$_2$, MnCl$_2$, CaCl$_2$ and EDTA (0, 2, 4, 6, 8 and 10 mM) were added to standard reaction. On the other hand, the enzymatic activity on different substrates was evaluated using 25 μM of 4MU-β-D-xylopyranoside, 4MU-glucoside, 4MU-β-D-cellobioside, 4MU-β-D-galactopyranoside, 4MU-β-D-glucopyranoside and 4MU-β-D-mannopyranoside in standard reactions. Additionally, kinetic parameters (K_m and V_{max}) were determined using increasing concentrations of 4MU-ABF (2.5, 5, 25, 50, 100 and 200 μm) in standard reactions. Analysis was performed through Lineweaver and Burk mathematic method.

Results

Fungal growth and extracellular ABF production by pathogenic and non-pathogenic races of *C. lindemuthianum*

When glucose was used as carbon source, a gradual growth of *C. lindemuthianum* was observed (Fig. 1A and B), pathogenic race reached 532.71 mg, while the non-pathogenic gotten 672.2 mg of dry wet at day 12 of incubation; nevertheless, no significant difference ($P<0.05$) was observed in the growth of both races of *C. lindemuthianum*. On the other hand, lower levels ABF extracellular activity was detected in both races, only day six of non-pathogenic race showed a significant value of activity (1.93 nM of 4-MU/min/μg of mycelium protein) (Fig. 1B).
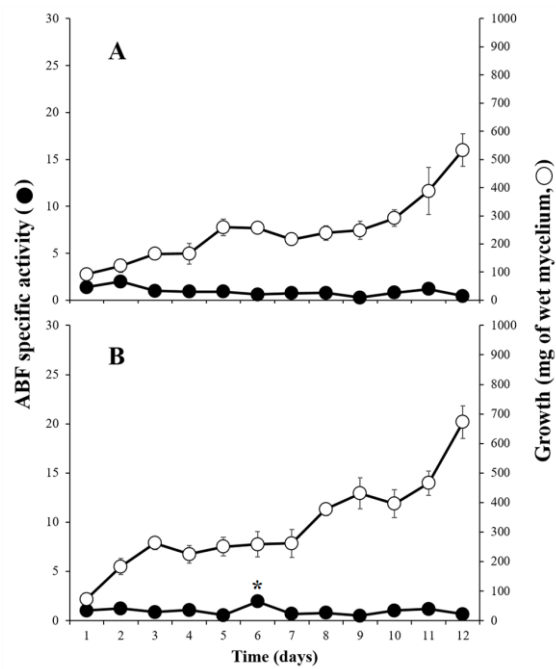

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Fig. 1 Growth and production of extracellular ABF activity by pathogenic (A) and non-pathogenic (B) races of *C. lindemuthianum* cultivated in the presence of glucose as the main carbon source. ABF specific activity is expressed in nM of 4-MU/min/µg of mycelium protein. Each point shows the mean of triplicates ± SE. The symbol "*" indicates significant changes ($P<0.05$) between races.

When glucose was replaced with xylan as main carbon source, no statistic difference ($P<0.05$) between races was detected during first seven days of growth, from 8th day, the non-pathogenic race grew faster than pathogenic race reached more than 2000 mg of dry wet at day 9-12 of growth and the pathogenic race gotten 1577.76 mg at day 12. These values represented about 30% more than the biomass produced with glucose in both races (Fig. 2A and B). Clear induction of ABF extracellular activity was observed in cultures supplemented with xylan. The non-pathogenic race showed higher levels of specific activity in most of the days, two higher peaks were detected after 5 and 10 days of growth (22. 58 and 19.76 nM of 4-MU/min/µg of mycelium protein respectively). On the other hand, levels of ABF extracellular activity in the pathogenic race showed two peaks at 5th and 8th days and a drastic increase at days 11 and 12 of induction of up to 19.67 and 25.59 nM of 4-MU/min/µg of mycelium, which is significantly higher than specific activity of non-pathogenic race in same days (Fig 2A and B).
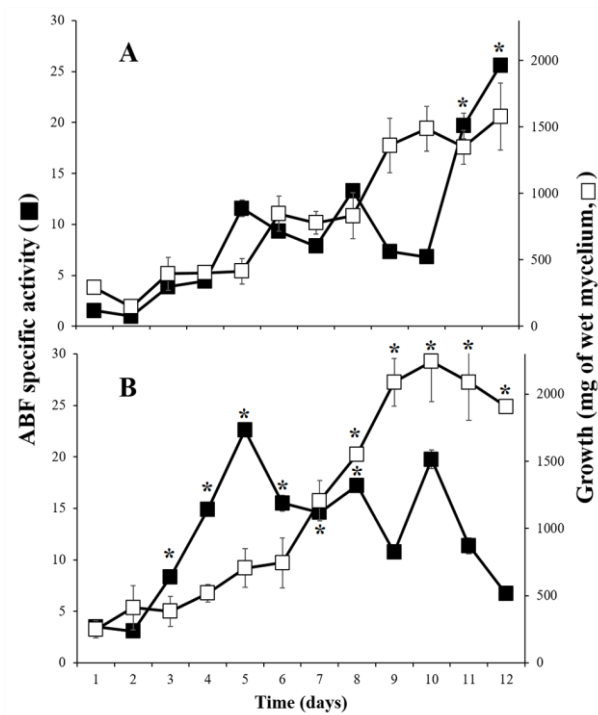

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Fig. 2 Growth and production of extracellular ABF activity by pathogenic (A) and non-pathogenic (B) races of *C. lindemuthianum* cultivated in the presence of xylan as the main carbon source. ABF specific activity is expressed in nM of 4-MU/min/µg of mycelium protein. Each point shows the mean of triplicates ± SE. The symbol "*" indicates significant changes ($P<0.05$) between races.

Arabinogalactan was also used as main carbon source, biomass values obtained in both races are similar to those observed in mycelium growth with glucose. Significant changes ($P<0.05$) between pathogenic and non-pathogenic races were observed in few times evaluated. Specific ABF activity in pathogenic race was increasing gradually from day 2 to 7 having a peak of activity at day 8 (13.70 nM of 4-MU/min/µg of mycelium protein) with a decrease in 9th day and a new increase to day 10 and 11 (Fig. 3A). On the other hand, ABF of non-pathogenic race showed two peaks of higher extracellular ABF activity. First peak was detected at day 3 of growth (7.57 nM of 4-MU/min/µg of mycelium protein) decreasing at day 4. Second peak was observed after 7th day, a rapid increase in specific activity level was detected with a maximum value at day 9 (14.38 nM of 4-MU/min/µg of mycelium protein) (Fig. 3B)
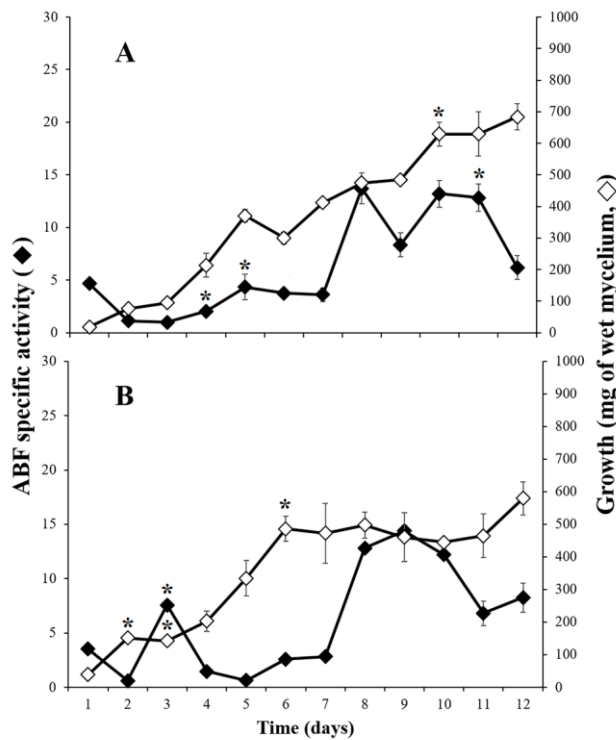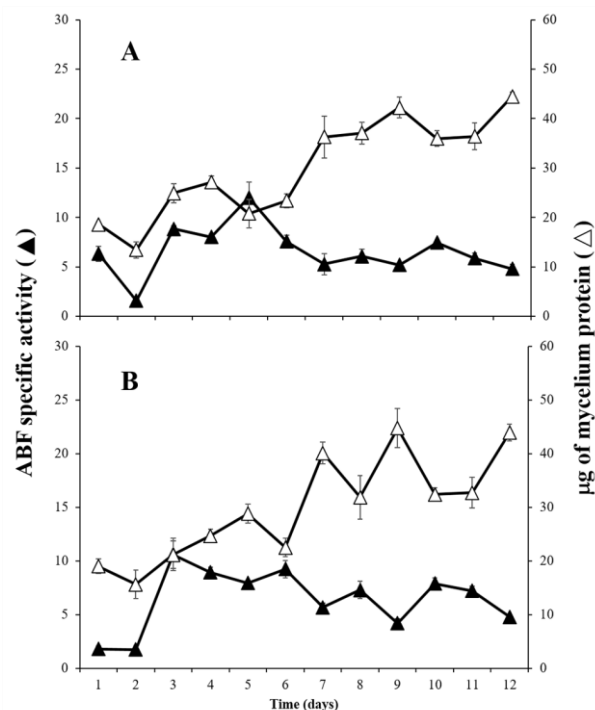

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Fig. 3 Growth and production of extracellular ABF activity by pathogenic (A) and non-pathogenic (B) races of *C. lindemuthianum* cultivated in the presence of arabinogalactan as the main carbon source. ABF specific activity is expressed in nM of 4-MU/min/µg of mycelium protein. Each point shows the mean of triplicates ± SE. The symbol "*" indicates significant changes ($P<0.05$) between races.

When Mathur medium was supplemented with plant cell walls from *P. vulgaris*, no significant differences were obtained in the mycelium protein values between pathogenic and non-pathogenic races of *C. lindemuthianum*. Interestingly, extracellular protein was detected since day one in both races with a rapid increase at day 7. Higher values in the amount of extracellular protein were detected at 9 and 12 days (39.72 and 43.7 µg/ml respectively) in pathogenic race and at 7th, 9th and 12th days (40.11, 44.72 and 43.9 µg/ml respectively) for non-pathogenic race (Fig. 4A and B). ABF specific activity in pathogenic race started at day one decreasing drastically at 2nd day, the maximum level of activity was reached at day 5 (11.98 nM of 4-MU/min/µg of mycelium protein) and constant values of activity were observed from day 7 to 12 (Fig. 4A). In non-pathogenic race, extracellular ABF activity increased drastically at 3rd day of growth with a maximum value of 10.6 nM of 4-MU/min/µg of mycelium protein (Fig. 4B).

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Fig. 4 Growth and production of extracellular ABF activity by pathogenic (A) and non-pathogenic (B) races of *C. lindemuthianum* cultivated in the presence of plant cell walls from *P. vulgaris* as the main carbon source. ABF specific activity is expressed in nM of 4-MU/min/μg of mycelium protein. Each point shows the mean of triplicates ± SE. The symbol "*" indicates significant changes ($P<0.05$) between races.

Purification of α-L-arabinofuranosidase from pathogenic race of *C. lindemuthianum*

With the purpose to obtain higher amounts of extracellular protein for ABF purification, a pre-inoculum from *C. lindemuthianum* race 1472 was growth in PD medium during 8 and 10 days, transferred to induction Mathur's medium supplemented with 2.5% of arabinogalactan and incubated during 6 days. ABF was purified to homogeneity; the result of the purification is summarized in Table 1. Filtered crude extract was concentrated ten times using Amicon Ultra Centrifugal Filters (Millipore). Concentrated extract was subject to previous desalt step increasing its specific activity 4.75-fold and two subsequent purification steps, a first one of chromatography of anionic exchange which showed a single peak with ABF activity (data not shown) (17.38-purification fold). Collected fractions from this peak were passed through exclusion molecular column and a single peak with ABF activity was obtained (91.79-purification fold).

Table 1 Purification chart of α-L-arabinofuranosidase from *C. lindemuthianum* race 1472.

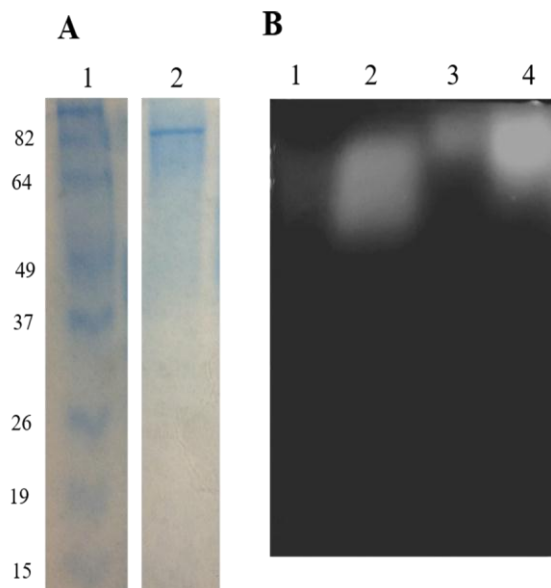| Purification step	Total activity (nM/min/ml)	Total protein (mg)	Specific activity (nM/min/mg)	Purification fold	Yield (%)
Crude extract	99.5	27.5	3.62	1	100
HiTrap Desalting	40.6	2.4	17.16	4.8	40.8
HiTrap Capto DEAE	18.3	0.29	62.8	17.4	18.4
HiLoad 16/600 Superdex	18.02	0.05	331.6	91.8	18.2

Fig. 5 A SDS-PAGE 10% stained with Coomassie R-250. Lane 1: BenchMark Protein Ladder. Lane 2: Purified ABF. **B** Zymogram showing ABF activity. Lane 1: concentrated desalt extract of fungal culture. Lane 2: fractions of anionic exchange. Line 3 and 4: purified ABF from C. *lindemuthianum* race 1472. The bands correspond to the hydrolysis of methylumbelliferyl α-L-arabinofuranoside.

SDS-PAGE showed that purified ABF from *C. lindemuthianum* race 1472 has an approximate molecular weight of 82 kD (Fig. 5A). On the other hand, Zymogram showed a single band with ABF activity (Fig. 5B), this results corroborates that the band observed in SDS-PAGE corresponds to ABF.

Enzymatic properties

The pH of the reactions was adjusted with two different buffers as described above. ABF has activity in a wide range of pH from 5-9 with an optimum pH of 6.0 (Fig. 6A). On the other hand, the enzyme was found to be stable in a range of temperature from 30-60ºC with an optimum temperature of 50ºC. A slight decrease in specific activity was observed at 70 and 80ºC, retaining about 80% of its activity at 80ºC after 45 min of incubation (Fig. 6B).
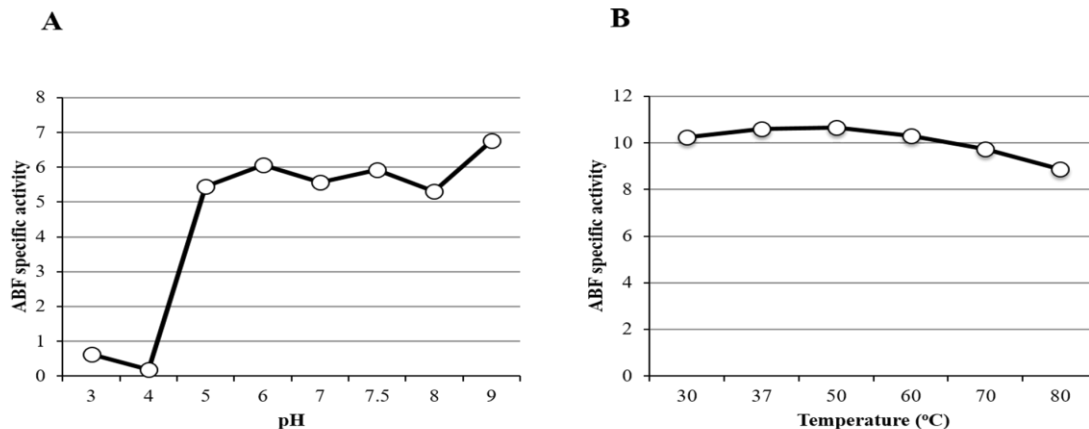
Fig. 6 A The effect of pH on ABF specific activity. The effect of pH was determined at 50ºC for 45 min. **B** The effect of temperature on ABF specific activity determined at pH 6 during 45 min. Specific activity is expressed as nM of 4MU/min/μg of mycelium protein.

The effect of the time of reaction incubation on ABF activity was also evaluated; higher enzymatic activity was detected after 20 min of incubation with a drastic decrease after 40 min and slightly decreases after 60 and 80 min. The enzyme retained about 20% of its enzymatic activity after 80 min of incubation (Fig. 7A). To analyze the effect of some cations and chelants on ABF activity, increasing concentrations of those were added to standard reactions (Fig. 7B). Both Mg^{2+} and Mn^{2+} have an effect on ABF activity; an increase on enzymatic activity was observed at 4 mM (310 and 200 nM of 4MU/min/μg of mycelium protein respectively) and decreasing in 6, 8 and 10 mM. When $CaCl_2$ was added to standard reactions, a strong increase of enzymatic activity was observed and the maximum activity was detected at 6 mM (486.7 nM of 4MU/min/μg of mycelium protein). Interestingly, the enzymatic activity was lowered when EDTA was added to enzymatic reactions (Fig. 7B). In agreement with these results ABF use Ca^{2+} as activator, but also can use Mg^{2+}, or Mn^{2+}.
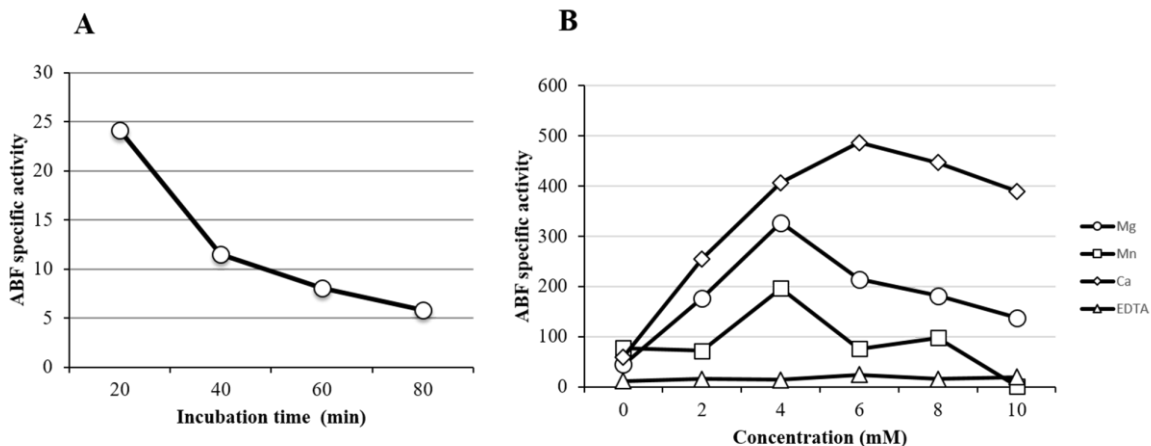
Fig. 7 A The effect of incubation time on ABF specific activity. The effect of time was determined at 50ºC and pH 6.

B The effect of cations and chelants on ABF specific activity determined at pH 6 at 50°C and 20 min. Specific

activity is expressed as nM of 4MU/min/µg of mycelium protein.

With the aim of evaluate the purified ABF specificity, enzymatic activity using other substrates was tested. Very

low enzymatic activity was detected on different substrates (0.22-7.82 nM of 4MU/min/µg of mycelium protein) in

comparison with enzymatic activity value obtained when 4-MU-ABF was used as substrate (86.53 nM of

4MU/min/µg of mycelium protein) (Fig. 8). These results suggest that purified ABF presents high substrate
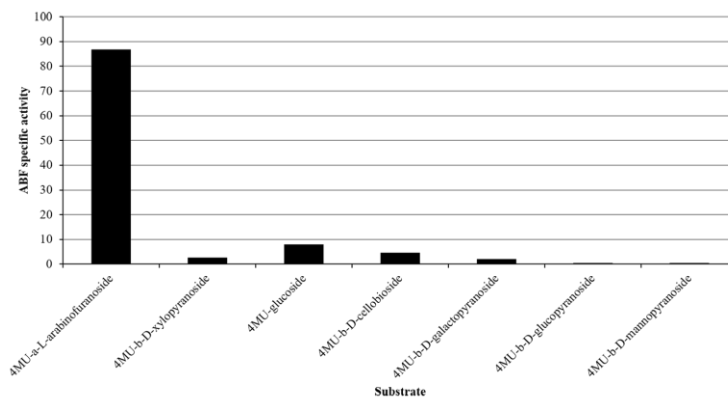
specificity.

Fig. 8 ABF specific activity on different substrates. Enzymatic activity was determined at 50ºC and 20 min of

incubation. Specific activity is expressed as nM of 4MU/min/µg of mycelium protein.

Finally, kinetic parameters were determined using increasing concentrations of 4MU-ABF as described above. Reciprocal plots (Fig. 9) displayed apparent K_m of 22.93 µM and V_{max} of 303.03 nM of 4MU/min/µg of mycelium protein.
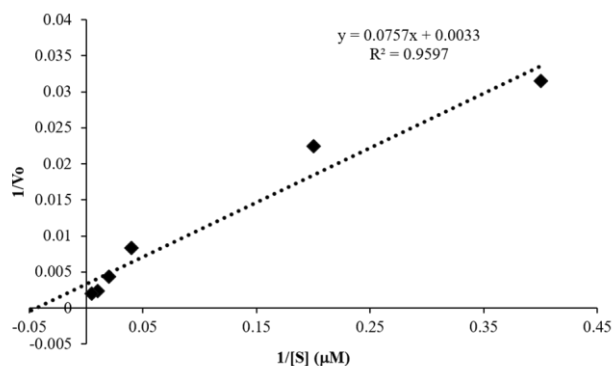

Fig. 9 Lineweaver-Burk plot of initial velocity data for ABF. The ABF activity was determined in 50 mM acetate buffer pH 6.0 at 50ºC during 20 min. V_o is expressed in nM of 4MU/min/µg of mycelium protein.

Discussion

Fungal growth and extracellular ABF production by pathogenic and non-pathogenic races of C. lindemuthianum

Lower levels of ABF enzymatic activity detected in cultures with glucose as carbon source observed in both races of *C. lindemuthianum* are agree with ABF enzymatic activity levels of *A. kawachii* evaluated in cultures supplemented with L-arabitol, L-arabinose, wheat bran, oat spelt xylan, D-xylose and D-glucose (Koseki et al. 2003). Additionally, basal levels of cellulase, pectin lyase (PNL2) and xylanase (XYL1) extracellular enzymatic activities were observed when glucose is used as substrate in cultures of *C. lindemuthianum* (Acosta-Rodríguez et al. 2005; Hernández-Silva et al. 2007). It has been reported that the expression of genes encoding hydrolytic enzymes is subject to catabolic repression through the action of CreA under a preferred carbon source (Strauss et al. 1995; Tani et al. 2014). Northern-blot analysis and Semi-quantitative RT-PCR analysis performed in *A. kawachii* and *P. chrysogenum* have showed that genes coding ABFs suffer catabolic repression in presence of glucose or sucrose (Koseki et al. 2003; Sakamoto et al. 2013).

An increase of ABF specific activity was observed when glucose was substituted with xylan, arabinogalactan or plant cell walls of *P. vulgaris* as main carbon source. Interestingly, xylan was the best substrate for growth of both

races of *C. lindemuthianum* and the best inductor of ABF specific activity. It has been reported that xylan or their degradation products like xylose or xylobiose are capable to induce the expression of genes coding hemicellulolytic enzymes including ABFs via XlnR transcription factor (Glass et al. 2013; Tani et al. 2014). Putative DNA-binding sites for Xlnr have been located in genes of ABFs (Fritz et al. 2008; Koseki et al. 2003), so higher levels of ABF production in both races of *C. lindemuthianum* in presence of xylan could be related to this phenomenon.

Hemicellulose degradation requires coordinated action of a battery of enzymes that depolymerize all its components in a manner coordinated (Conejo-Saucedo et al. 2011), endo-xylanases, β-xylosidases and α-L-arabinofuranosidases acts synergistically to complete hydrolysis of arabinoxylan (Lagaert et al. 2014). Xylanase extracellular production was evaluated in both races of *C. lindemuthianum* in cultures supplemented with xylan as main carbon source, higher levels of enzymatic activity were detected at first seven days of growth (Conejo-Saucedo et al. 2016), xylanase action liberates arabino-xylooligosaccharides, which are then hydrolyzed by arabinofuranosidases and β-xylosidases (Lagaert et al. 2014), Al least two arabinofuranosidases have been purified in *Aspergillus* sp (de Vries_2001). Recently, degradation regionselectivities of GH51 and GH54 ABFs were studied; GH51 ABFs removed arabinosyl residues from internally and terminal non-reducing end of xylopyranosyl residues with mono and disubstitutions, showing ABF-m/d activity and versatility in substrate specificity (Koutaniemi and Tenkanen 2016). It is possibly that an increase in ABF production after five days of growth in both races on *C. lindemuthianum* occurs once arabino-xylooligosaccharides are released.

Although the levels of extracellular production of ABF in presence of arabinogalactan are lower than with xylan, it was possible to detect higher induction of ABF activity after seven days of incubation. It has been reported that α-L-arabinofuranosidases are essential for arabinogalactan complete hydrolysis (Sakamoto and Ishimaru 2013). Probably, degradation products of arabinogalactan (arabinose or galactose), might be able to induce the expression of genes that coding ABFs.

Finally, when plant cell walls from *P. vulgaris* were used as main carbon source, important levels of secreted protein were detected since day one. Basal levels of polysaccharidases are necessary to start cell walls degradation, unlike the enzymatic activity measured in cultures with xylan or arabinogalactan, the higher levels of ABF specific activity were observed during the first days of growth in both races of *C. lindemuthianum.*

Purification and biochemical characterization of ABF from *C. lindemuthianum*

α-L-arabinofuranosidases are debranching enzymes with biological and biotechnological importance; nevertheless, no works about ABFs have been reported in *Colletotrichum* genus. The ABF purified of *C. lindemuthianum* represents the first report of biochemical purification and enzymatic properties determination of a hydrolytic enzyme in *C. lindemuthianum*.

The approximate molecular weight calculated by SDS-PAGE (~82 kDa) is similar to molecular weight (70-85 kDa) of other native ABFs purified in fungi, specifically those located in family 51 of glycoside hydrolases. Biochemical characterization of ABF showed that the enzyme is active in a wide range of pH (5-9) and temperature (30-80ºC), retaining up to 80% of enzymatic activity, this result is similar to optimal pH and temperature reports to GH51 ABFs purified from other fungi (pH 4-5; temperature 50-60ºC) (Flipphi et al. 1994; Fritz et al. 2008; Kaneko et al. 1998; Sakamoto and Kawasaki 2003; vd Veen et al. 1991). ABF activity is higher at 20 min of incubation it lost about 80% of its activity after 80 min of incubation. Divalent cations addition to the enzymatic reactions increases the ABF activity, particularly Ca^{2+}, and the EDTA abates the activity. Calcium is an important component in the cell wall structure, this cation is found in ranges from 10 μM to 10 mM cross-linking carboxylic residues of pectins, in short stretches of de-methoxylated homogalacturonans, providing rigidity to the wall (Hepler and Winship 2010; Picton and Steer 1983). So, pectin degradation releases Ca^{2+}, which is an activator of ABF, similar to the Pectin lyases depend on Ca^{+2} (Lara-Márquez et al. 2011). Considering this result in the standard enzymatic reactions, ABF used Na^{2+} from the sodium acetate buffer instead of Ca^{2+}, but also can use Mg^{2+} or Mn^{2+}.

On the other hand, ABF from *C. lindemuthianum* showed high substrate specificity, low enzymatic activity on other substrates was detected, suggesting high specificity on α-links and low or null activity on β-links. Stereoselectivity and regionselectivity of ABFs and other hydrolytic enzymes have been previously described (Koutaniemi and Tenkanen 2016).

K_m value calculated to ABF from *C. lindemuthianum* on 4-MU-ABF (0.23 mM) is in agreement with values of K_m in other fungi, similar value was reported to *P. capsulatum* (0.18 mM) (Filho et al. 1996).

The wide range of temperature and pH which ABF has enzymatic activity suggest that this protein could be a good candidate to a biotechnological application.

In conclusion the pathogenic and non-pathogenic races of *C. lindemuthianum* are able to synthesize and secrete ABF activity when grown under different culture conditions. Xylan was the best inductor of growth and extracellular ABF

activity for both races. The ABF purified of *C. lindemuthianum* race 1472 has wide range of pH and temperature, is

activated by divalent cations, mainly Ca^{2+}, and has high substrate specificity which make it in a good candidate to be

applied in biotechnology and industrial process.

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VII. DISCUSIÓN GENERAL

El género *Colletotrichum* se considera como uno de los géneros de hongos fitopatógenos más relevantes de acuerdo a su importancia económica y científica (Dean et al., 2012). Particularmente, *C. lindemuthianum* ha sido un modelo interesante en el estudio de la interacción planta-patógeno gracias a su estilo hemibiotrófico de nutrición-infección y la amplia diversidad de patotipos que presenta (Perfect et al., 1999; Rodríguez-Guerra et al., 2006). De la misma manera que otros hongos fitopatógenos, *C. lindemuthianum* secreta una variedad enzimas hidrolíticas que le permiten la degradación de los polímeros de la pared celular vegetal, obteniendo de esta manera el carbono necesario para su sobrevivencia (Anderson, 1978). Debido al papel tan importante que juegan las enzimas hidrolíticas durante el proceso de infección, estas han sido propuestas como factores de virulencia (Villa-Rivera et al., 2017); adicionalmente, en los últimos años estas proteínas han cobrado gran relevancia debido a su aplicación biotecnológica y a su incorporación eficiente en varios procesos industriales (Kirk et al., 2002).

Como parte del sistema hemicelulolítico de hongos, se encuentran la endo-β-(1,6)-D-galactanasa (EBG) y la α-L-arabinofuranosidasa (ABF), ambas enzimas participan en la hidrólisis de las ramificaciones de arabinogalactanos (AG) tipo II, actuando de manera sinergística y coordinada. Particularmente, la EBG solo es capaz de hidrolizar ramificaciones donde los terminales en arabinosa hayan sido previamente removidos por una arabinofuranosidasa (Sakamoto & Ishimaru, 2013). Por otra parte, la actividad arabinofuranosidasa en relevante no solo para la degradación de AGs, la arabinosa se encuentra asociada a las cadenas de hemicelulosa, por lo que la acción de esta enzima junto con xilanasas y β-xilosidasas permite la despolimerización completa de arabinoxilanos (Lagaert et al., 2014). Debido a que no existen trabajos previos dentro del género *Colletotrichum* para estas dos enzimas, el presente trabajo representa un enfoque novedoso en el estudio de estas dos proteínas, además de aportar información para el posible desarrollo de un producto biotecnológico.

Como primera parte de este estudio, se realizó el aislamiento y caracterización de los genes codificantes de ambas enzimas (*ebg* y *abfA*) a partir de ambas razas (patógena y no patógena de *C. lindemuthianum*). En el caso del gen *ebg*, fue posible secuenciar tanto el ADN genómico como el ADNc. Al localizar este gen en los genomas de otras especies de *Colletotrichum* disponibles en bases de datos fue posible identificar en la mayoría de las especies una copia del mismo, lo cual

es congruente con estudios de southerblot realizados para *T. viride* donde se identificó una sola copia del gen *ebg* (Kotake et al., 2004). Sin embargo, en los genomas de *C. orbiculare*, *C. higginsianum* y *C. sublineola* se localizaron dos copias del gen *ebg*.

Por otra parte, se obtuvo la secuencia de ADN genómico que codifica para una α-L-arabinofuranosidasa, la comparación con bases de datos mostró porcentajes altos de similitud e identidad con arabinofuranosidasas de la familia 51 de las glicosil hidrolasas (GH). En especies de *Aspergillus* se han reportado la existencia de por lo menos dos α-L-arabinofuranosidasas, las cuales pertenecen a las familias GH51 y GH54 respectivamente (Koseki et al., 2003), se ha encontrado la presencia de un gen para la ABF de la GH51 (ABFA o ABF1) y de hasta cuatro genes para la ABF de la GH54 (ABFB o ABF2) (Guais et al., 2010), consistente con lo anterior, una sola copia del gen *abfA* fue localizada en todos los genomas de *Colletotrichum* disponibles en bases de datos.

La secuencia de nucleótidos de los genes *ebg* como *abfA* para la raza patógena y no patógena de *C. lindemuthianum* presentaron 100% de identidad, este resultado es congruente con el análisis realizado de los genes codificantes de la pectin liasa (*Clpnl2*) y la β-1,4-endoxilanasa (*xyl1*), donde al comparar las secuencias obtenidas a partir de ambas razas de *C. lindemuthianum*, no se observó ningún cambio a nivel de nucleótidos (Conejo-Saucedo et al., 2016; Lara-Márquez, Zavala-Páramo, López-Romero, Calderón-Cortés, et al., 2011). De la misma forma, al comparar las secuencias de nucleótidos codificante de la ABFA localizadas en los genomas de dos cepas brasileñas de *C. lindemuthianum* (83.501 y 89) disponibles en bases de datos, se obtuvo el 100% de identidad. Sin embargo, al realizar la comparación de las secuencias obtenidas en este trabajo con las secuencias de *C. lindemuthianum* 83.501 y 89, fue posible localizar algunos cambios a nivel de nucleótidos, y al realizar el análisis de la secuencia deducida de aminoácidos se obtuvo el 99% de identidad y 100% de similitud. Por lo anterior, es posible hipotetizar que los pequeños cambios observados en las secuencias de nucleótidos sugieren diferenciación debido a la distancia geográfica que existe entre patotipos mexicanos y brasileños, más que por su grado de virulencia.

A partir de las secuencias deducidas de aminoácidos fue posible realizar el modelaje tridimensional de la EBG y la ABF. Debido a que no existe una EBG o ABFA cristalizada en hongos para utilizar como templado, los modelos generados presentan valores de calidad bajos. En el caso particular de la ABFA, existen proteínas cristalizadas aisladas de bacterias, sin

embargo, los porcentajes de similitud con la ABFA de *C. lindemuthinum* son muy bajos (18% de identidad y 21% de similitud). Se ha reportado que la ABFA de la GH51 de bacterias es una estructura multimérica formada por seis monómeros, cada uno de ellos con dos dominios (barril $(\beta/\alpha)_8$ y β-sandwich con topología jelly-roll) (Hövel et al., 2003), sin embargo, no existe evidencia de que esto mismo ocurra en hongos.

A pesar de que no se encontraron cambios en las secuencias de nucleótidos de los genes *ebg* y *abfA* entre la raza patógena y no patógena, sí se observó una clara diferencia en el perfil de expresión de ambos genes. Los niveles de expresión de la raza patógena son, en la mayoría de las condiciones evaluadas, mayores que los de la raza no patógena. Esta diferencia en el perfil de expresión entre la raza patógena y no patógena ha sido observada también para los genes *Clpnl* y *xyl1* de *C. lindemuthianum* (Conejo-Saucedo et al., 2016; Lara-Márquez et al., 2011). Adicionalmente, es posible observar que la expresión de los genes *ebg* y *abfA* es coordinada, esto podría deberse a que la actividad α-L-arabinofuranosida y endo-β-(1,6)-D-galactanasa en la degradación de AGs tipo II es sinergística y coordinada también. La despolimerización eficiente de AGs tipo II que están unidos a proteínas (AGPs), podría constituir un paso clave en el establecimiento de la infección, se ha reportado que las AGPs podrían estar involucradas en la percepción de estímulos causantes de las respuestas de defensa debido a que son proteínas de señalización de la pared celular (Jamet et al., 2008; Jamet et al., 2006). Aunque no se conoce con claridad su mecanismo de señalización, se han propuesto algunos modelos para explicar la manera en la que se podría llevar a cabo la comunicación y/o adhesión celular (Showalter, 2001). Por otro lado, se ha demostrado que algunas AGPs presentan un dominio conservado de seis residuos de cisteína denominado dominio PAC (prolina, AGP, cisteína) con una pequeña glicoproteína LAT52 que se ha reportado que interactúa con un receptor RLK (receptores de cinasas) llamado LePRK2. Se ha sugerido que el dominio PAC es el intermediario en la unión entre AGPs y RLKs y así mismo se plantea que RLKs y AGPs pueden ser el enlace entre la matriz de polisacáridos y la señalización celular (Seifert, 2007). El componente carbohidrato de las AGPs es fundamental para ésta o cualquier otra función, se ha reportado que si este es degradado, las AGPs pierden parcial o completamente su función (Mashiguchi, 2008). Por lo anterior, es posible hipotetizar que la degradación de AGs podría ser un factor importante en el establecimiento de la infección para la raza patógena pero no para la no patógena; lo cual podría explicar las diferencias en el perfil de expresión observado para ambos genes.

Por otra parte, en análisis de la región regulatoria identificada en los genes *ebg* y *abfA* de especies de *Colletotrichum* mostró que ambos genes tienen varias secuencias putativas de unión al factor de transcripción para represión catabólica CreA. A pesar de que en este análisis no se incluyeron secuencias de la raza 0 y 1472 de *C. lindemuthianum*, para el análisis del promotor del gen *abfA,* se incluyó la secuencia del promotor del gen *abfA* identificado en el genoma de la raza 89 de *C. lindemuthianum,* donde se identificaron 12 sitios putativos de unión a CreA. Dado que el perfil de expresión del gen *abfA* en micelio crecido con glucosa muestra niveles basales del transcrito de *abfA,* es posible que estemos observando un fenómeno de represión catabólica vía CreA unido a secuencias de ADN identificadas en el promotor de *C. lindemuthianum.* Por otra parte, se localizaron algunos sitios de unión a XnlR, el factor de transcripción involucrado en la expresión de genes de varias celulasas y hemicelulasas (Glass et al., 2013). Sin embargo, solo nueve promotores de los 15 genes *ebg* analizados presentaron al menos una secuencia de unión para este factor de transcripción, en el caso de los promotores de genes *abfA* analizados, ocho contienen una secuencia de unión a XnlR y en ocho no se identificó algún sitio putativo de unión, incluyendo el promotor de la *abfA* de *C. lindemuthianum* raza 89. Por otro lado, en los promotores de ambos genes se localizaron sitios putativos de unión a otros factores de transcripción como PacC, Gal4 y AceI, particularmente en promotores del gen *abfA*, fue posible localizar hasta 26 sitios putativos de unión para el factor de transcripción AraR o factor GATA, el cual está involucrado en la remodelación de la cromatina (Battaglia et al., 2011; Muro-Pastor et al., 1999). Los resultados del análisis del promotor del gen *abfA* son congruentes con los de análisis similares reportados para otros genes de ABFs de la GH51, donde se han identificado de uno a 12 sitios de unión a CreA, de uno a dos para PacC, al menos uno para XnlR y AceI y hasta siete sitios para AreR (Fritz et al., 2008). Estos análisis sugieren que los genes *ebg* y *abfA* de especies de *Colletotrichum* podrían estar bajo la regulación de factores de transcripción involucrados en la regulación de la expresión de genes de enzimas xilanolíticas y celulolíticas. Es necesario contar con las secuencias de los promotores de los genes *ebg* y *abfA* de la raza patógena y no patógena de *C. lindemuthianum* para identificar secuencias putativas de unión a estos factores de transcripción.

Además de evaluar el perfil de expresión del gen *abfA* en respuesta a diferentes inductores, se determinó el crecimiento y la actividad extracelular ABF en cultivos de *C. lindemuthianum* suplementados con las mismas fuentes de carbono (glucosa, xilana, arabinogalactano y pared

celular de *P. vulgaris*). El crecimiento de *C. lindemuthianum* fue mayor en la cultivos donde se utilizó xilana como fuente de carbono tanto en la raza patógena como no patógena, alcanzando los valores máximos de 2242.45 y 1577.77 mg respectivamente, después del día 10 de incubación. El crecimiento de la raza 0 fue significativamente mayor que el de la raza 1472 a partir del día 8 de incubación. El crecimiento micelial en cultivos con glucosa y arabinogalactano para ambas razas fue muy similar, en cultivos supplementados con glucosa se obtuvieron los valores máximos de crecimiento en el día 12 de 532.72 y 672.2 mg para la raza patógena y no patógena respectivamente. Para cultivos con arabinogalactano como fuente de carbono se obtuvieron valores de 683.25 mg en la raza 1472 y 572.62 mg para la raza no patógena, en ambos sustratos, no se encontró diferencia estadista significativa en los valores de crecimiento obtenidos en las dos razas de *C. lindemuthianum*. Debido a la dificultad para separar la pared celular del micelio, no fue posible evaluar el crecimiento del hongo en cultivos con pared celular vegetal.

En cultivos supplementados con glucosa la actividad extracelular ABF fue basal, de nuevo esto es congruente con el fenómeno de represión catabólica de genes que codifican enzimas involucradas en la degradación de la pared celular vegetal al estar en presencia de la fuente preferente de carbono, la glucosa (Tani et al., 2014). Al sustituir la glucosa con la xilana, arabinogalactano y pared celular, se observó la inducción de la actividad ABF. Aunque no se observó diferencia estadística significativa entre la raza 0 y 1472 en la mayoría de las condiciones, la actividad enzimática de ABF fue mayor en cultivos de la raza 0 crecidos con xilana. Aunque los resultados de actividad enzimática de ABF no son congruentes con el perfil de expresión del gen *abfA* evaluado en este trabajo, donde la expresión del gen en la raza patógena es mayor que en la raza no patógena; se debe considerar que para la determinación de actividad enzimática no es posible establecer con exactitud cuántas proteínas con actividad ABF están contribuyendo en los niveles de actividad enzimática que se están detectando. Se sabe que en hongos existen al menos dos proteínas con actividad α-L-arabinofuranosidasa, la ABFA y la ABFB (de Vries & Visser, 2001; Koseki et al., 2003), mientras que en el perfil de expresión solo se evaluaron los niveles del transcrito de *abfA*. Por otra parte, el mejor inductor de la actividad ABF fue la xilana, se ha establecido que los productos de degradación de la xilana (xilana y xilobiosa) son capaces de inducir la expresión de genes que codifican enzimas hidrolíticas vía el factor de transcripción XnlR (Glass et al., 2013).

Debido a que las a-L-arabinofuranosidasas son enzimas capaces de degradar selectivamente componentes de la pared celular vegetal, se les ha utilizado en procesos biotecnológicos e industriales (Numan & Bhosle, 2006), sin embargo, no existen reportes de la purificación de esta proteína para el género *Colletotrichum*. En el presente trabajo se reporta la purificación de una ABF de *C. lindemuthianum* raza 1472, lo cual constituye el primer reporte de la purificación y caracterización de una proteína para esta especie.

El peso molecular de la ABF purificada de *C. lindemuthianum* calculado por SDS-PAGE fue de ~82 kDa, lo cual es congruente con el peso molecular de otras ABFs de la familia GH51 purificadas en hongos (70-85 kDa) (Tabla 4). La caracterización bioquímica de la ABF mostró que la enzima es activa en un rango amplio de pH que va de 5 a 9 y una temperatura de 30°C a 80°C conservando hasta un 80% de su actividad enzimática. Estos resultados son congruentes con los valores de pH (4-5) y temperatura óptima (50-60°C) reportados para otras ABFs de hongos (Tabla 4). El rango de pH en el que la ABF es activa incluye tanto pHs ácidos como básicos, aunque se ha establecido que las enzimas líticas de hongos funcionan óptimamente a pHs ácidos, se ha reportado que los productos de degradación de las mismas llevan a cabo una alcalinización gradual del ambiente de interacción (Prusky et al., 2001), lo que podría explicar su actividad en pHs básicos. La actividad de ABF es mayor después de 20 minutos de incubación, disminuyendo gradualmente a los 40, 60 y 80 minutos conservando solo el 20% de actividad después de 80 minutos. Interesantemente, la adición de cationes divalentes (principalmente Ca^{+2}) a las reacciones enzimáticas incrementó la actividad ABF, mientras que al adicionar un quelante como EDTA abatió casi por completo la actividad enzimática. El calcio es un componente estructural de la pared celular vegetal, se encuentra en rangos de 10 µM a 10 mM unido al componente carboxilo en residuos de pectina, confiriéndole rigidez a la pared (Hepler & Winship, 2010; Picton & Steer, 1983). De esta manera, la degradación de pectina libera Ca^{+2}, que podría ser un activador de ABF, similar a lo que ocurre con las pectin liasas dependientes de Ca^{+2} (Lara-Márquez et al. 2011). Por lo anterior, en las reacciones enzimáticas estándar, la ABF utiliza Na^{+2} obtenido del buffer de acetato en lugar de Ca^{+2}, pero también puede utilizar Mg^{+2} o Mn^{+2}.

Por otro lado, la ABF de *C. lindemuthianum* mostró alta especificidad por sustrato, los niveles bajos de actividad enzimática detectados en los sustratos evaluados (diferentes al 4-MU-ABF) sugiere alta especificidad en enlaces α y actividad baja sobre enlaces β, se ha demostrado las

enzimas hidrolíticas y particularmente las ABFs poseen una estéreo-selectividad y regio-selectividad muy alta (Koutaniemi & Tenkanen, 2016).

Las propiedades bioquímicas de la ABF como el rango amplio de pH y temperatura en el que es activa, sugiere que esta enzima podría ser un buen candidato para aplicaciones biotecnológicas. Adicionalmente, en contar con una actividad ABF de *C. lindemuthianum* permitirá evaluar la actividad endo-β-(1,6)-D-galactanasa, la cual es activa solo en sustratos desarabinosilados, lo cual aportará información importante acerca de la degradación coordinada de arabinogalactanos tipo II.

VIII. PERSPECTIVAS

1. La cristalización de la endo-β-(1,6)-D-alactanasa y de la α-L-arabinofuranosidasa permitiría el conocimiento y descripción precisas de la estructura tridimensional de ambas proteínas, y de esta manera, realizar análisis de docking para evaluar la interacción de las proteínas con el sustrato.

2. Con el fin de realizar el análisis de la región regulatoria de los genes *ebg* y *abfA* es necesario obtener las secuencias correspondientes a partir de la raza patógena y no patógena de *C. lindemuthinaum*

3. Evaluar la producción extracelular de la EBG aportaría información relevante acerca de su actividad coordinada con otras enzimas de *C. lindemuthianum*. Para lo cual es necesario también la elaboración de un sustrato cromogénico que permita evaluar la acción de la enzima.

4. Purificar y caracterizar bioquímicamente la EBG a partir de *C. lindemuthianum*.

5. La expresión heteróloga de la EBG y la ABF y la purificación y caracterización de proteínas recombinantes supondrá la obtención de una mayor cantidad de proteína necesaria para aplicaciones biotecnológicas y/o procesos industriales.

6. El silenciamiento de ambos genes nos aportaría información acerca del papel de las mismas en el establecimiento de la enfermedad.

IX.LITERATURA CITADA COMPLEMENTARIA

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