Centro de Investigación en Alimentación y Desarrollo, A.C.

RECONOCIMIENTO MOLECULAR DE FRAGMENTOS DE QUITINA FÚNGICA EN FRUTO DE TOMATE Y SU INDUCCION DEL MECANISMO DE TRADUCCION DE SEÑAL

Por:

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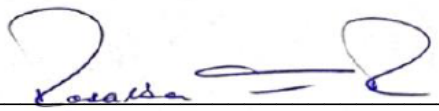

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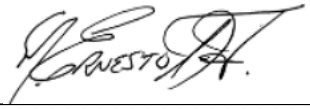

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CONTENIDO

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RESUMEN

Las enfermedades fúngicas en postcosecha de frutas y hortalizas representan uno de los factores importantes que provocan pérdidas económicas en la industria alimentaria. El tomate es un cultivo económicamente importante, siendo susceptible al ataque de hongos como *Alternaria alternata*. Para el control de este fitopatógeno se han propuesto alternativas al uso de fungicidas sintéticos, como la inducción del mecanismo de defensa natural, el cual puede ser activado en respuesta al reconocimiento de moléculas específicas del fitopatógeno, como la quitina. Se ha demostrado que la quitina y sus oligosacáridos inducen la respuesta de defensa en plantas; sin embargo estudios sobre su efecto en frutos son escasos. El objetivo de esta investigación fue evaluar los genes que codifican para receptores de membrana en frutos de tomate expuestos a los fragmentos de quitina de *A. alternata,* así como su nivel de expresión. Se extrajeron fragmentos de quitina de *A. alternata*, se caracterizaron parcialmente y aplicaron sobre frutos de tomate, tomando muestras del pericarpio a diferentes tiempos posteriores a la exposición (pae). Se determinó la capacidad elicitora de los fragmentos mediante la actividad enzimática de la quitinasa y el nivel de expresión del gen que codifica para la quitinasa (*Chi* 1). Se determinó la expresión diferencial de genes que codifican a los receptores de quitina mediante RNA-Seq. Se obtuvieron fragmentos de quitina con peso molecular ≤ 1 kDa, grado de polimerización estimado de <5, grado de acetilación del 76.7 %, y contenido de N-acetil glucosamina de 33.6 µg/mL. Los fragmentos de quitina indujeron una mayor actividad de la quitinasa a los 30 min pae, así como una elevada sobreexpresión relativa del gen *Chi 1*, a ese mismo tiempo. Los resultados del RNA-Seq indicaron la sobreexpresión de genes ortólogos que codifican a receptores tipo CERK1 involucrados en el reconocimiento de quitina, como *Solyc01g098420.3,* y *Solyc07g049180.3* (con alto porcentaje de similitud con los genes que codifican a los receptores Bti9 y SILYK1*), Solyc02g089900.1* (con alto porcentaje de similitud con el gen que codifica al receptor tipo LYK4), *Solyc07g049180 y Solyc02g089900* (genes homólogos que codifican al receptor Bti9). Se encontraron, además, genes que participan en la síntesis de moléculas de señalización (etileno y ácido jasmónico) y genes involucrados en el mecanismo de defensa del fruto. Se concluye que los fragmentos de quitina de *A. alternata* inducen la sobrexpresión de genes que codifican para receptores de quitina en fruto de tomate, así como de genes involucrados en el mecanismo de defensa.

Palabras clave: fragmentos de quitina fúngica, fruto de tomate, *A. alternata*, RNA-Seq, Expresión diferencial.

ABSTRACT

Postharvest fungal diseases of fruits and vegetables represent one of the important factors that cause economic losses in the food industry. Tomato is an economically important crop, being susceptible to attack by fungi such as *Alternaria alternata*. For the control of this pathogen, alternatives to the use of synthetic fungicides have been proposed, such as the induction of the natural defense mechanism, which can be activated in response to the recognition of specific molecules of the phytopathogen, such as chitin. Chitin and its oligosaccharides have been shown to induce the defense response in plants; however, studies on its effect on fruits are scarce. The objective of this research was to evaluate the genes that code for membrane receptors in tomato fruits exposed to chitin fragments of *A. alternata*, as well as their level of expression. Chitin fragments from *A. alternata* were extracted, partially characterized and applied to tomato fruits, taking samples from the pericarp at different times after exposure (pae). The elicit capacity of the fragments was determined by the enzymatic activity of chitinase and the expression level of the gene that codes for chitinase (*Chi 1*). The differential expression of genes encoding chitin receptors was determined by RNA-Seq. Chitin fragments with molecular weight ≤ 1 kDa, estimated polymerization degree of <5, acetylation degree of 76.7 %, and N-acetyl glucosamine content of 33.6 μg/mL were obtained. The chitin fragments induced a higher chitinase activity at 30 min pae, as well as a high relative overexpression of the Chi 1 gene, at the same time. The RNA-Seq results indicated the overexpression of orthologous genes that encode CERK1-like receptors involved in the recognition of chitin, such as *Solyc01g098420.3*, and *Solyc07g049180.3* (with a high percentage of similarity with the genes that code the *Bti9* and *SILYK1*), *Solyc02g089900.1* (with a high percentage of similarity with the gene that codes the LYK4-like receptor), *Solyc07g049180* and *Solyc02g089900* (homologous genes that code the *Bti9* receptor). In addition, genes that participate in the synthesis of signaling molecules (ethylene and jasmonic acid) and genes involved in the defense mechanism of the fruit were found. It is concluded that chitin fragments from *A. alternata* induce the overexpression of genes that code for chitin receptors in tomato fruit, as well as genes involved in the defense mechanism.

Key words: fungal chitin fragments, tomato fruit, *A. alternata*, RNA-Seq, differential gene expression

1. SINOPSIS

1.1 Justificación

Las pérdidas postcosecha de frutas y hortalizas a nivel mundial son alarmantes. En los países en desarrollo, en donde existe una gran deficiencia en la infraestructura de mercado, las pérdidas ascienden a un 55% (Kitinoja y Kader, 2015; Porat *et al*., 2018). Esto representa una pérdida significativa de alimentos y un considerable daño económico para los países productores. Una de las causas fundamentales es el ataque por fitopatógenos fúngicos como *Alternaria alternata, Botrytis cinerea, Colletotrichum gloeosporioides, Fusarium oxysporum,* entre otros (Manzo *et al*., 2016; Alkan y Fortes, 2015; Troncoso-Rojas y Tiznado-Hernández, 2014; Takao *et al*., 2016).

Las pérdidas de frutas y verduras por pudrición pueden representar hasta el 20-25%, desarrollando los síntomas de la enfermedad regularmente durante el almacenamiento (Dukare *et al*., 2018). Entre los frutos afectados que son de gran relevancia por su importancia económica y elevado consumo, se encuentra el tomate. El tomate es un fruto altamente perecedero y posee corta vida de anaquel (Suslow y Cantwell, 2003). Además, debido a su alto contenido de agua y fisiología, es altamente susceptible al hongo necrótrofo *A. alternata,* causante de la enfermedad conocida como pudrición negra (Troncoso-Rojas y Tiznado-Hernández, 2014). Durante el almacenamiento refrigerado, las condiciones de temperatura y humedad relativa alta, en combinación con el estado de madurez del fruto, crean un ambiente favorable para el desarrollo de la enfermedad (Prusky *et al*., 2013).

El método tradicional empleado para el control de estas enfermedades es la utilización de productos químicos. Sin embargo, este método se ha tratado de erradicar en los últimos años debido a que representan un riesgo para el medio ambiente y la salud humana, por la posible residualidad química en los alimentos (llevando a la inseguridad en el consumidor), y por el desarrollo de cepas resistentes (Dukare *et al*., 2018; Gupta, 2017). Debido a lo anteriormente planteado existe una tendencia hacia el consumo de orgánicos por parte de la población (Carvalho *et al*., 2012).

Con el fin de plantear estrategias de control de enfermedades en postcosecha basadas en la resistencia inducida, que garanticen la inocuidad alimentaria y que a su vez disminuyan los porcentajes de pérdidas, resulta importante generar conocimiento que permita elucidar como ocurre

el fenómeno del reconocimiento de hongos fitopatógenos por parte de los frutos. Debido a esto, se propone la necesidad de evaluar moléculas de la pared celular de *A. alternata,* tales como la quitina y oligosacáridos de quitina, así como su participación en la transducción de señales que activan las respuestas de defensa en frutos de tomate.

Se ha demostrado en los últimos años que la quitina es esencial en el reconocimiento planta-patógeno (Buendia *et al*., 2018; Malik *et al*., 2020), así como en la activación del mecanismo de defensa natural en las plantas (Pusztahelyi, 2018). Se ha reportado en tejido vegetativo la presencia de receptores que reconocen oligosacáridos de quitina, lo cual provoca la activación de las vías de señalización, así como las respuestas de defensa, como la activación de la cascada de las MAP cinasas, la generación de EROs (Especies reactivas de oxígeno), la inducción de proteínas relacionadas a la patogénesis (PR) (Iizasa *et al*., 2010), la activación de enzimas involucradas en la síntesis de fitoalexinas, entre otras, que participan en la protección de la planta/fruto de los fitopatógenos.

El reconocimiento de oligosacáridos de quitina ha sido ampliamente estudiado y caracterizado en plantas de *Arabidopsis thaliana* y *Oryza sativa*. Sin embargo, la información publicada sobre receptores de quitina con dominios de lisina que activa la respuesta inmune en fruto tomate es muy limitada. La información científica que existe actualmente está relacionada a los receptores de quitina que reconocen moléculas de quitina sintética (obtenida por síntesis química), o de origen marino, o de levadura. Además, otros estudios se refieren a los genes que codifican a receptores en respuesta a la infección por fitopatógenos, o en respuesta a micorrizas arbusculares, pero se desconoce la respuesta de frutos de tomate a la exposición de oligosacáridos de quitina aislados de un hongo fitopatógeno, como es *A. alternata*. Por lo anterior, en la presente tesis se propone identificar en frutos de tomate los genes ortólogos que posiblemente estén involucrados en el reconocimiento de quitina de *A. alternata* y su posible participación en las rutas de señalización y activación del mecanismo de defensa. Los resultados que se generen contribuirán a elucidar el fenómeno de reconocimiento de quitina fúngica y esclarecer el mecanismo de defensa del fruto que se activa en respuesta a estrés biótico.

1.2 Antecedentes

1.2.1 Calidad y Pérdidas Postcosecha en Tomate (*Solanum lycopersicum*).

El tomate es una de las hortalizas más consumida en todo el mundo y la de mayor valor económico, siendo China el país de mayor producción con más de 60 millones de toneladas producidas en 2018 (FAOSTAT, 2020). Este fruto representa uno de los componentes más frecuentes de la dieta humana, debido a su notable riqueza en vitaminas, azúcares, compuestos antioxidantes, pigmentos carotenoides, microelementos, metabolitos secundarios, sales minerales y fibras. Posee, además, excelentes cualidades organolépticas, que mejoran el apetito y ayudan a la digestión de los alimentos (Alarcón, 2013).

La demanda de fruto de tomate aumenta continuamente y con ella su cultivo, producción y comercio. México es uno de los principales productores de tomate a nivel mundial y el primer exportador hacia Estados Unidos. Según la FAOSTAT, en el año 2018 se cosecharon 4,559,375 ton (8vo. lugar a nivel mundial) en una superficie de 90,323 ha, con un rendimiento de 50.47 ton de tomate por ha cosechada. Por concepto de valor de producción se obtuvieron $1’545,570,311 de USD en ese mismo año (FAOSTAT, 2020).

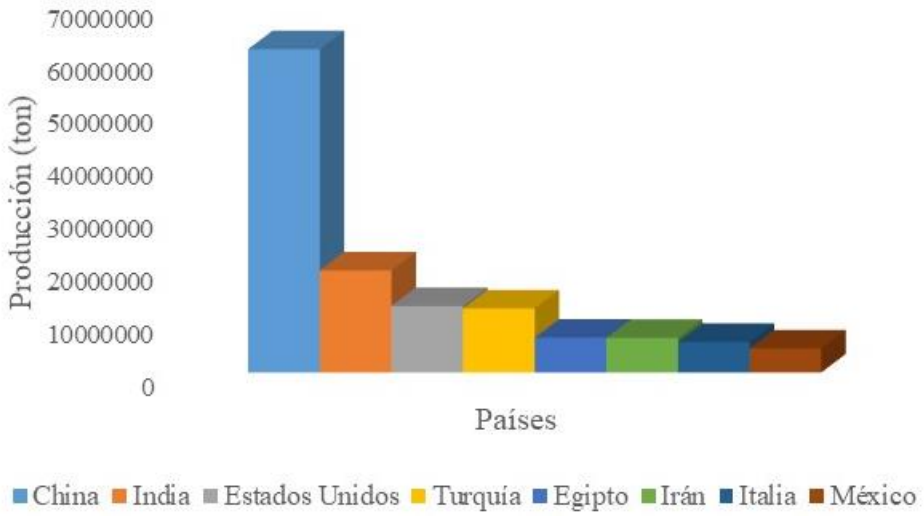

Figura 1. Producción mundial de tomate en el año 2018 (FAOSTAT, 2020).

La calidad del tomate fresco es un aspecto importante en la comercialización, la cual está determinada por la industria agroalimentaria en base a la forma, color, tamaño, acidez y libre de daños visuales, como heridas y pudriciones (Salas, 2014). La producción de tomate de mala calidad afecta la economía de la industria agroalimentaria, por lo que es importante realizar un manejo adecuado desde la cosecha hasta que llega al consumidor para conservar su excelente calidad.

La madurez del tomate al momento de ser cosechado es un componente integral de la calidad que determina su vida de anaquel y afecta la forma en que debe ser manipulado, transportado y comercializado (Alam *et al*., 2006). Durante la maduración del tomate, se producen cambios importantes en el color, composición, aroma, y textura. Dentro de estos cambios ocurre la síntesis de licopeno que imparte al fruto el color rojo y la degradación de clorofila, así como la síntesis de ácidos orgánicos, azúcares y compuestos volátiles característicos del aroma para los diferentes estados de madurez. Todas estas transformaciones, unidas a una disminución en la firmeza del epicarpio, hacen al fruto atractivo para el consumo humano (San Martín-Hernández *et al*., 2012).

El fruto de tomate es altamente perecedero y posee una corta vida de anaquel. Cuando se almacena el tomate en estado verde maduro a una temperatura de 12.5 a 15°C, presenta una vida útil de 14 días; mientras que para un fruto en estado de madurez rojo almacenado entre 7 y 10°C, su vida útil es de 3 a 5 días. Estas condiciones de temperatura, humedad relativamente alta y estado de madurez (de rosa a rojo maduro) lo convierten en un fruto altamente susceptible al ataque por diversos patógenos (Prusky *et al.*, 2013).

Los principales agentes causales de la pudrición son los hongos, los cuales son conocidos por causar considerables pérdidas en postcosecha de frutas y hortalizas (Ruiz-Martínez *et al.*, 2012). En México, las pérdidas postcosecha se encuentran entre un 10 y un 50%, de las cuales el 28% pertenecen al ataque por patógenos fúngicos (Kitinoja y Kader, 2015). El fruto de tomate es altamente susceptible a diversos hongos necrótrofos entre los que se destacan *Rhizopus nigricans Ehrenb* (Pan *et al*., 2013)*, Fusarium oxysporum* (Manzo *et al*., 2016), *Botrytis cinerea* (Alkan y Fortes, 2015), *y A. alternata* (Troncoso-Rojas y Tiznado-Hernández, 2014; Takao *et al*., 2016).

A. alternata es el agente causal del moho negro, siendo esta enfermedad una de las más importantes durante el almacenamiento refrigerado de tomate. Su importancia no sólo radica por las pérdidas económicas que produce a nivel postcosecha de frutos y vegetales, también se considera peligroso para el ser humano ya que produce micotoxinas como alternariol, monometiléter, altenueno, ácido L-tenuazónico y alterotoxinas, entre otros (Lee *et al*., 2015). Además, provoca alergia (Gabriel *et al*., 2016), asma (Dang *et al*., 2015), y queratitis fúngica en humanos (Xu *et al*., 2013).

1.2.2. *Alternaria alternata* y su Patogenicidad Durante Postcosecha en Tomate

A. alternata es un hongo filamentoso, saprófito, perteneciente al filo *Ascomycota* y al grupo de los dematiáceos, caracterizados por presentar una coloración oscura. Es la especie de hongo más común en los productos agrícolas, la especie está definida como saprofita de alimentos, pero se encuentra principalmente en frutos y vegetales. Se ha reportado su presencia en distintas partes del mundo, lo que nos señala que puede adaptarse a distintos tipos de climas (Troncoso-Rojas y Tiznado-Hernández, 2014).

Según su morfología, las colonias de *A. alternata* son color verde oliva y poseen un margen blanco con un tamaño en el rango de 2-5 mm cuando se desarrollan en agar papa dextrosa. A los 7-10 días de crecimiento, las colonias aumentan a más de 70 mm de diámetro. Forma cadenas de entre 6 y 14 conidios de longitud, que pueden volver a ramificarse y formar cadenas terciarias de dos u ocho conidios, estos poseen una longitud de 20-50 µm de largo y tienen forma ovalada (Figura 2). Las hifas septadas y conidióforos aparentan un color marrón claro (Lawrence *et al*., 2013).

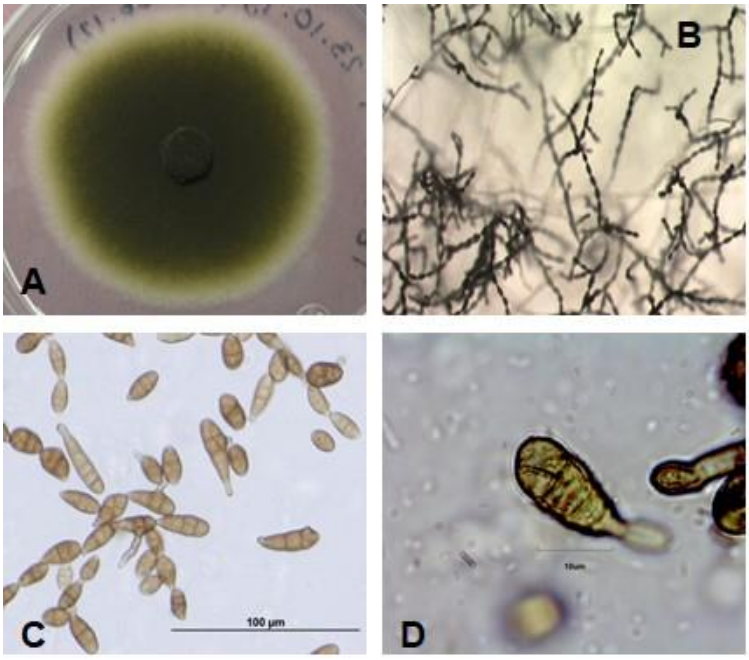

Figura 2. Morfología de *A. alternata*. **A**, Colonia de *A. alternata* en agar Papa Dextrosa. **B**, Cadena de conidias y tipo de ramificación de *A. alternata*. **C**, Conidias a 40X. **D**, Conidia a 100X. (Fotografías B y C proporcionadas por el Profesor Barry Pryor, Plant Pathology, Universidad de Arizona, Tucson, Arizona, USA.)

La esporulación de *Alternaria* es óptima a 27°C pero es inhibida por debajo de 15°C o por encima de 33°C, aunque el rango de crecimiento de este hongo está entre 0 y 35°C. La actividad de agua mínima para el desarrollo de este hongo es 0.88 y la óptima casi 1.00% (Carrillo, 2003). Este microorganismo posee una fase patogénica en la cual puede infectar a diversas especies vegetales, siendo especialmente susceptibles los tejidos estresados, débiles, viejos o heridos (Laemmlen, 2002; Sinavimo, 2018). La infección por *A. alternata* sucede por la adhesión de la espora a la cutícula y el crecimiento del tubo germinal se da sobre la planta. Sus hifas se expanden por la degradación de las glicoproteínas y polisacáridos presentes en la pared celular como glucanos y quitina. Al germinar los conidios, se forman un tubo germinal y un apresorio (Troncoso-Rojas y Tiznado-Hernández, 2014). De este último surgen unas hifas especializadas llamadas clavijas de penetración, las cuales se introducen en la cutícula y pared celular. La penetración se favorece debido a la acumulación de melanina en su pared celular. Este pigmento oscuro (melanina) beneficia la penetración, ya que produce una capa rígida, los solutos son atrapados por el apresorio e induce la entrada de agua al mismo, aumentando su presión de turgencia y facilitando la introducción en el tejido vegetal (Troncoso-Rojas y Tiznado-Hernández, 2014).

A. alternata es descrito como un patógeno débil y oportunista, que permanece en estado latente hasta que el tejido madure y se den las condiciones de temperatura y humedad relativa para que se lleve a cabo la infección (Prusky *et al*., 2013). En frutos de tomate, la infección se caracteriza por lesiones en la superficie del fruto maduro (Figura 3). Las lesiones son concéntricas, de un color café claro a oscuro afectando solamente la epidermis de los frutos (Snowdon, 2010).

Figura 3. Lesiones provocadas por *A. alternata* en frutos de tomate (Imágenes tomadas en el laboratorio de Biotecnología de Vegetales y Poscosecha).

El desarrollo de la infección se favorece por daños mecánicos, quemaduras solares, o daño por frío

(Snowdon, 2010). Estos daños ocurren durante la cosecha, el transporte o almacenamiento del fruto, por lo que se puede concluir que las pérdidas postcosecha en tomate, en su mayoría, ocurren durante estas etapas (Pane *et al*., 2016).

1.2.3. Control del Deterioro Fúngico

1.2.3.1 Métodos de control convencionales. La conservación de la calidad del tomate demanda la aplicación de diversos métodos, entre los que destaca el uso de fungicidas sintéticos para el control de la pudrición por *A. alternata*, a saber: Clorotalonil, Captafol, Azoxystrobin (Prusky, 1996), sec-Butilamina, Imazalil, Procloraz, Benomilo, Tiabendazol y Captan 80 WG (Herrera *et al*, 2011; Troncoso-Rojas y Tiznado-Hernández, 2014). Sin embargo, se ha comprobado que el uso indiscriminado de estos químicos, no solo ha creado resistencia por parte de las poblaciones de hongos, sino que con el uso prolongado y excesivo han provocado niveles crecientes de residuos en los productos hortofrutícolas, así como contaminación en el suelo aumentando los riesgos de salinización (Alarcón, 2013; Gupta, 2017).

Con el fin de disminuir los daños causados por los fungicidas químicos hay un creciente interés público y científico hacia el desarrollo de nuevos productos basados en estrategias más novedosas, ecológicas y menos costosas, que garanticen la inocuidad de los alimentos. Una posible alternativa para reducir o inhibir el desarrollo de fitopatógenos, es la inducción del mecanismo de defensa natural (Malinovsky *et al*., 2014).

1.2.4 Métodos Alternativos: Inducción del Mecanismo de Defensa Natural

La relación perjudicial que existe entre las plantas y microorganismos patógenos ha llevado a las plantas al desarrollo de un sistema de defensa. Este sistema de defensa está basado en la prevención, tolerancia o resistencia (Thakur, 2007); los cuales se activan de diferente manera, de acuerdo con el tipo de patógeno que esté atacando a la planta (necrótrofo o biótrofo). Estos mecanismos de defensa pueden ser constitutivos, es decir, mecanismos de defensa presentes en la planta que proveen de forma pasiva resistencia contra patógenos; mientras que los mecanismos inducidos se

activan solamente como una respuesta al ataque de patógenos (Malik *et al.*, 2020).

Las defensas constitutivas o preexistentes incluyen barreras físicas y químicas preformadas, que son estructuras o sustancias químicas presentes en la planta antes de la infección del patógeno. Entre las defensas estructurales se pueden mencionar las ceras de la cutícula, el espesor y firmeza de las células de la epidermis, tricomas, el tamaño y la forma de estomas y lenticelas, que actúan como barreras dificultando la penetración de algunos patógenos (Hématy *et al*., 2009).

Por otro lado, las defensas químicas son compuestos secundarios que se encuentran presentes en la planta antes de que se lleve a cabo la infección, son de origen diverso con alta actividad biocida. Las plantas secretan una amplia variedad de sustancias con propiedades tóxicas o inhibidoras como fenoles, flavonoides, lignina, taninos, antocianinas, saponinas, glucosinolatos y lectinas. Tambien ciertas enzimas relacionadas a la patogénesis (PRs) como glucanasas y quitinasas, entre otros actúan como defensas químicas (Agrios, 2005).

Los mecanismos de defensa pueden ser inducidos por diferentes tipos de estrés de origen abiótico y biótico. En el caso del estrés biótico, las defensas se activan como respuesta al ataque por fitopatógenos, provocando cambios en el metabolismo de la planta. Este fenómeno se conoce como resistencia inducida, en el cual la reacción de hipersensibilidad (RH) es la máxima expresión de resistencia en las plantas, y se define como una muerte rápida de las células vegetales asociada con la restricción del crecimiento del patógeno, (Broekgaarden, 2015).

La RH se activa una vez que las células epidérmicas han sido invadidas y penetradas por algún fitopatógeno y se caracteriza por lesiones necróticas en el tejido que pueden desarrollarse en las primeras 24 h de la interacción debido entre otras causas a una variación del flujo de iones y fosforilación (Asai y Yoshioka, 2009). Las células hipersensibles ubicadas al interior de la epidermis, al detectar al organismo fitopatógeno inducen su muerte celular programada (MCP). Este es un mecanismo utilizado por algunos hospederos con la finalidad de evitar la diseminación del patógeno intra o extracelularmente, además en ocasiones llegan a originar la muerte del patógeno. Posteriormente la planta sintetiza compuestos como lignina y compuestos antimicrobianos con el fin de resguardar la zona afectada (Sanzón y Savaleta, 2011). En respuesta a estos eventos se activan diversas rutas metabólicas que alertan a la planta, promoviendo de manera coordinada la síntesis de enzimas relacionadas a la patogénesis como quitinasas, glucanasas, peroxidasas, así como otras enzimas involucradas en la síntesis de fitoalexinas, como la fenilalanina amonio-liasa, la cual es una enzima clave en la síntesis de esos compuestos (Alkan

y Fortes, 2015; Pandey *et al*., 2016).

Actualmente se han reportado dos formas de resistencia inducida: la resistencia sistémica adquirida (RSA, por sus siglas en español; o SAR, por sus siglas en inglés) y la resistencia sistémica inducida (RSI, por sus siglas en español; o ISR por sus siglas en inglés), las cuales se distinguen dependiendo de la naturaleza del elicitor y de las rutas de señalización que participan durante la interacción planta-patógeno. La resistencia sistémica adquirida se puede definir como la resistencia a subsecuentes ataques por patógenos y se puede detectar en partes de la planta infectada y no infectada, teniendo una íntima relación con la RH. La SAR, dentro del mecanismo de transducción de señales, no necesariamente obedece la interacción gen a gen; una vez que se activa el mecanismo la resistencia se activa por toda la planta. Posteriormente esa información se transfiere a lo largo y ancho de la planta vía floema, contra atacando a una amplia variedad de fitopatógenos por un tiempo que va desde algunas semanas hasta meses (Matilla, 2018).

La SAR e ISR presentan características que permiten diferenciarlas. SAR es inducida por un amplio espectro de elicitores bióticos o abióticos que inducen proteínas relacionadas a la patogénesis (PR, por sus siglas en español), utiliza rutas de señalización en las cuales puede estar involucrado el ácido salicílico, y la señal viaja sistémicamente a sitios lejanos de donde ocurrió la infección. Por su parte, la ISR se ha relacionado con rizobacterias promotoras del crecimiento (PGPR), en las que no se involucra la participación de proteínas PR, y la ruta de señalización se realiza a través del ácido jasmónico y etileno (Choudhary *et al*., 2007).

1.2.5 Moléculas Fúngicas que Estimulan el Mecanismo de Defensa Natural

Cuando las plantas son infectadas, ocurren varios cambios en la cutícula, los cuales causan la activación de las respuestas de defensa (Stael *et al.,* 2015). Una vez que los fitopatógenos vencen esa primera línea de defensa, inicia la segunda línea, la cual está basada en la inmunidad innata de las células que les permite resistir o bloquear a los patógenos (Kushalappa *et al*., 2016).

El paso esencial en la activación de los mecanismos de defensa es la detección de microorganismos mediante receptores de membrana conocidos como patrones de receptores de reconocimiento (PRRs, por sus siglas en inglés), los que reconocen a los patrones moleculares altamente

conservados. Entre estos patrones se encuentran los patrones moleculares asociado a microorganismos (MAMPs, por sus siglas en inglés), y los patrones moleculares asociados a patógenos (PAMPs, por sus siglas en inglés). Estas moléculas son secretadas por microorganismos, o liberadas de su pared celular por enzimas hidrolíticas durante la interacción con la planta. Entre las moléculas consideradas como MAMPs se encuentran principalmente la flagelina, lipopolisacáridos (LPs), factor de elongación (EF-Tu), y peptidoglucanos (de Wit, 2007); mientras que la quitina es considerada como PAMP (Pandey *et al.*, 2016). La percepción de MAMPs/PAMPs durante la infección por patógenos desencadena reacciones de defensa conocido como inmunidad desencadenante (PTI, PAMP-triggered immunity por sus siglas en inglés). Sin embargo, los patógenos han desarrollado mecanismos para producir factores de virulencia o efectores, que suprimen la PTI (Inmunidad activada por PAMP) para facilitar la patogénesis. En respuesta, las plantas han adquirido receptores adicionales, conocidos como proteínas de resistencia (R), que reconocen efectores de patógenos para inducir una respuesta llamada inmunidad activada por efectores (ETI) que finalmente desencadena la muerte de las células en las plantas (Pandey *et al.*, 2016). En ese sentido, MAMPs y PAMPs se consideran como moléculas inductoras capaces de inducir una respuesta de defensa en las plantas (Trouvelot *et al.*, 2014).

El reconocimiento celular de MAMPs o PAMPs ocurre por los receptores de membrana, conocidos como patrón de receptores de reconocimiento (PRRs, por sus siglas en inglés), dando lugar a la activación de respuestas de defensa a través de las cascadas de señalización que le permiten a la planta responder a un amplio rango de estímulos (Figura 4). Entre estas rutas se señalización, se encuentran las proteínas activadas por mitógenos (MAP cinasas), y proteínas cinasas dependientes de calcio, las cuales envían un mensaje para la producción de hormonas de señalización como ácido salicílico (AS), ácido jasmónico (AJ), etileno (ET), ácido abscísico (ABA), auxinas (AUX), citocininas (CK), giberelinas (GB), y brasinoesteroides (BR). La principal función de estas moléculas de señalización consiste en transducir los estímulos extracelulares reconocidos por receptores de la célula a un gran número de moléculas blanco, que en completa coordinación integran respuestas intracelulares altamente específicas al estímulo externo agresor (Broekgaarden, 2015). Además, se inducen cambios fisiológicos como el cierre de estomas que limitan la entrada del patógeno, canales aniónicos de Cl- y NO-, fosfolípidos transmembranales, la producción de especies reactivas de oxígeno, óxido nítrico, y la formación de depósitos de calosa (Matilla, 2018). Como parte de esas respuestas, se induce la expresión de genes de defensa en el núcleo. Dichos

genes codifican para proteínas PRP (Proteínas relacionadas a la patogénesis) como las glucanasas, quitinasas, peroxidasas, entre otras, así como enzimas involucradas en la síntesis de fitoalexinas (como fenoles, flavonoides, terpenoides, saponinas, entre otros) (Alkan *et al*., 2015; Pandey et *al*., 2016). En este sentido, un mecanismo de defensa exitoso inhibirá el desarrollo de la enfermedad y limitará el daño a la planta.

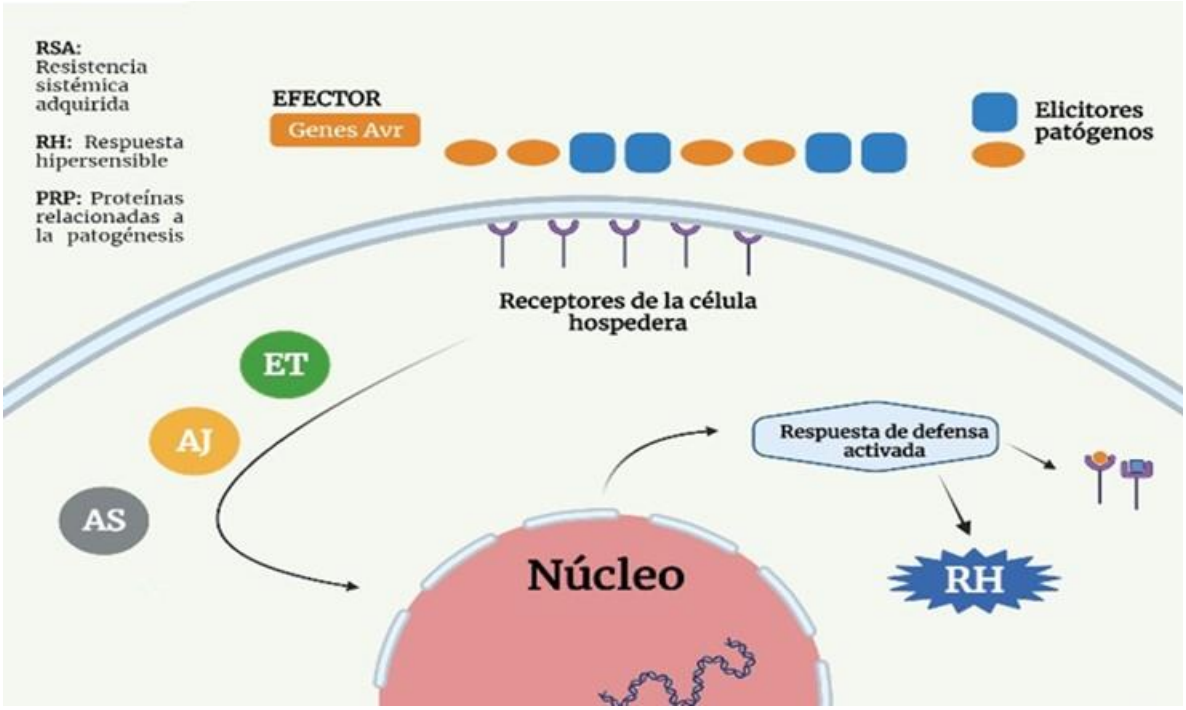

Figura 4. Mecanismo de interacción planta-patógeno (Modificado de la fuente Sánchez-Vallet et. al., 2014).

La defensa natural de la planta puede ser activada por elicitores o activadores físicos, químicos y biológicos. Los elicitores de tipo biológico han sido utilizados en forma muy activa, tal es el caso del uso de fragmentos de pared celular de hongos para inducir la transcripción de genes de defensa que se expresan durante las interacciones planta-patógeno (Egusa *et al*., 2008). Otros estudios han aplicado cepas no virulentas de microorganismos saprófitos o patogénicos que inducen la resistencia sistémica adquirida en el tejido vegetativo hospedero y en frutos postcosecha, como en manzana (De Capdeville *et al.*, 2003), zanahorias (Mercier *et al.*, 2000), toronja (Droby *et al.*, 2002), pera (Tian *et al.*, 2006), entre otras. Evidencias experimentales obtenidas en nuestro

laboratorio demuestran que la aplicación de un elicitor derivado de *Fusarium oxysporum*, indujo un aumento en la actividad de enzimas relacionadas con la defensa del melón Cantaloupe, así como una reducción significativa del desarrollo de *F. oxysporum* en los frutos (Sanchez-Estrada *et al*., 2009). Resultados similares se obtuvieron al aplicar el bioelicitor para el control del mismo hongo en frutos de tomate, observando un incremento en la síntesis de ácidos fenólicos y flavonoides, así como una reducción del 85% en el desarrollo del patógeno en tomate (Troncoso-Rojas *et al*., 2013). Estudios recientes reportaron la aplicación de quitina de levadura para activar el mecanismo de defensa y controlar la infección fúngica en el fruto. Zhang *et al*., (2016) observaron en frutos de pera que al aplicar quitina de *Rhodotorula mucilaginosa* indujo la expresión diferencial de proteínas como glucosidasas, hidrolasas, NADH desidrogenasas y quinasas con dominios de serina/treonina, además de una reducción significativa en el desarrollo de la enfermedad causada por *Rhizopus stolonifer*. En otro estudio realizado en frutos de tomate, se observó que al aplicar quitina de *Sacharomyces cerevisiae* se indujo un incremento en la expresión de enzimas del metabolismo oxidativo, como superóxido dismutasa, peroxidasa y catalasa. Asimismo, se observó un incremento en la actividad de proteínas relacionadas a la patogénesis como glucanasa y quitinasa (Sun *et al*., 2018).

1.2.5.1 Reconocimiento celular de quitina. La pared celular fúngica se compone de glicoproteínas, β-glucanos y quitina, la cual induce la activación de la respuesta de defensa de plantas frente a patógenos fúngicos. La quitina está compuesta de unidades de N-acetilglucosamina las cuales están unidas por enlaces β-1,4 (Figura 5). Microscópicamente, se encuentra en forma cristalina o semicristalina (Pillai *et al*., 2009), lo que convierte a este polisacárido en un material rígido y resistente que actúa como barrera muy potente en la pared celular o cutícula y protege a los propios organismos (Geoghegan *et al*., 2017). Aunque la quitina es insoluble cuando es extraída de fuentes marinas por medio de concentraciones elevadas de NaOH y temperatura (Malerba y Cerana, 2019), la extracción de quitina de origen fúngico por medio de tratamientos enzimáticos en combinación con sonicación, ha permitido la obtención de oligosacáridos de quitina parcialmente soluble en agua (Henry *et al*., 2019). De acuerdo con los estudios realizados por Buendía *et al*., (2018) y Roy *et al*., (2017), se ha demostrado que sus oligosacáridos son solubles en función del pH y su grado de polimerización.

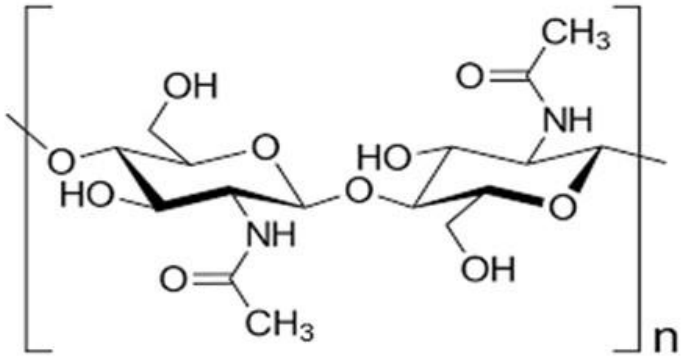

Figura 5. Estructura de la quitina (Tomado de la fuente Castañeda *et al*., 2011).

A nivel de reconocimiento molecular, diversos estudios señalan la relación de la quitina y sus oligosacáridos con la inducción del mecanismo de defensa en plantas. Asimismo, se ha reportado que el reconocimiento de estos oligosacáridos es mucho más efectivo dependiendo del grado de polimerización. Se ha comprobado que entre 5 y 8 unidades de polimerización es más efectivo mientras que a grados menores no se ha encontrado afinidad por los receptores de membrana (Zhang *et al*., 2002; Liu *et al*., 2012). Se ha demostrado que el oligosacárido de quitina con un grado de polimerización de ocho es el más activo entre los oligosacáridos de quitina para la activación de respuestas relacionadas con la defensa (Kuchitsu *et al*., 1997). Asensio *et al*., (2000) al realizar ensayos de afinidad entre oligosacáridos de quitina y dominios conservados de unión a quitina del árbol de corcho (*Hevea brasiliensis*), observaron una relación lineal de 1:1 para $(GlcNac)_5$ con el receptor, y para $(GlcNac)_8$ la relación fue (2:1) proteína-oligosacárido, lo cual demuestra que es probable una dimerización de receptores para el reconocimiento de los oligosacáridos de quitina, dependiendo de su tamaño. En otro estudio, Zhang *et al*., (2002) demostraron en *Arabidopsis thaliana* que oligosacáridos con un grado de polimerización entre 6 y 8 fueron reconocidos por receptores de membrana. Estos resultados fueron similares a los reportados por Iizasa *et al*., (2010), quienes observaron que oligosacáridos de quitina con grado de polimerización entre 5 y 9 también fueron reconocidos por receptores de membrana en *Arabidopsis*. Por otra parte, Petutsching *et al.,* (2010) reportaron que oligosacáridos con un grado de polimerización bajo no mostraron afinidad con receptores de membrana en *Arabidopsis.*

1.2.5.2 Receptores de membrana vegetal que reconocen oligosacáridos de quitina. Evidencias científicas demuestran que los miembros de familias de receptores tipo cinasas (RLK, por sus siglas en inglés) de algunas plantas y receptores tipo proteínas (RLP, por sus siglas en inglés) que contienen 3 dominios de lisina (LysM) extracelulares, se unen directamente y/o participan en la percepción de oligosacáridos de quitina (Buendia *et al*., 2018). Se ha reportado además que los receptores RLK poseen dominios internos que se enlazan a proteínas citoplásmicas como la PBL 27, que serán las primeras en fosforilarse y activar la ruta de las MAP cinasas (Yamada *et al*., 2016).

En las plantas, los RLK constituyen una familia de proteínas cinasas involucradas en diversos aspectos del crecimiento y desarrollo de las plantas, como la expansión celular, el desarrollo reproductivo, la resistencia a enfermedades, las respuestas al estrés abiótico, entre otros (He y Wu, 2016). Según Shiu *et al*., (2004), se han identificado más de 610 RLK en el genoma de *Arabidopsis*. Según sus estructuras de dominio extracelular (ECD), las RLK se clasifican en 44 subfamilias (Beck *et al*., 2012; Monaghan *et al*., 2012; Muthamilarasan y Prasad, 2013). En el arroz, las RLK se han expandido a una familia de cinasas con al menos 1131 miembros (Shiu *et al*., 2004). Se ha demostrado que los miembros de familias de RLK de algunas plantas y RLP que contienen 3 dominios de LysM extracelulares, se unen directamente y / o participan en la percepción de oligosacáridos de quitina (Buendia *et al*., 2018).

Los RLK son un grupo de proteínas de membrana con una región extracelular con un dominio sensor, una región transmembrana, y una región intracelular con dominios de serina/treonina con homología a proteínas cinasas involucradas en la transmisión de señales. Estos receptores se dividen en subfamilias de acuerdo con la cantidad de residuos de lisina en su región extracelular (He y Wu, 2016). Por otra parte, los RLP no tienen un dominio intracelular y en algunos casos son anclados a la membrana por medio de un dominio transmembranal de otra proteína o a un grupo glicosil-fosfatidil inositol (Macho y Zypfel, 2014; Buendía *et al*., 2018, Malik *et al*., 2020).

Estos receptores de membrana del tipo RLK o RLP que reconocen la quitina han sido ampliamente estudiados y caracterizados en *Oryza sativa* (arroz) y *Arabidopsis thaliana*. En las plantas de arroz, la proteína de unión al inductor de quitina (CEBiP) fue el primer receptor de quitina identificado, contiene dominios de lisina (LysM) que reconocen la quitina fúngica (Kaku *et al*., 2006). El peso molecular de esta proteína se estimó en 75 kDa y tiene 11 sitios de glicosilación a través de los cuales se puede unir N-acetilglucosamina (NAcGlc). Según algunos autores, en la planta de arroz

hay dos receptores implicados en la inmunidad activada por quitina (Shimizu *et al*., 2010). La proteína de unión inductora de quitina LysM-RLP (OsCEBiP) se une a fragmentos de quitina N-acetilados, iniciando la homodimerización del receptor y una heterodimerización con OsCERK1. Esta formación de heterotetrámeros desencadena PTI inducida por quitina (Hayafune *et al*., 2014). Además, los autores demostraron que en el receptor OsCEBiP, el motivo de lisina central es esencial en el reconocimiento de oligosacáridos de quitina con un grado de polimerización de 8. CEBiP se dimeriza con la proteína OsCERK1 para unirse a estos oligosacáridos. En este sentido, el sistema receptor de quitina en arroz requiere tanto CEBiP como OsCERK1 para la detección y señalización de quitina. En otro estudio, Shinya *et al*., (2015) afirmaron que dos moléculas receptoras de OsCEBiP se unen simultáneamente a uno de los oligosacáridos de quitina, interactuando con los grupos de N-acetilglucosamina que se encuentran en los residuos internos de GlcNAc y forman un homodímero, activando diversas respuestas inmunitarias (Figura 6).

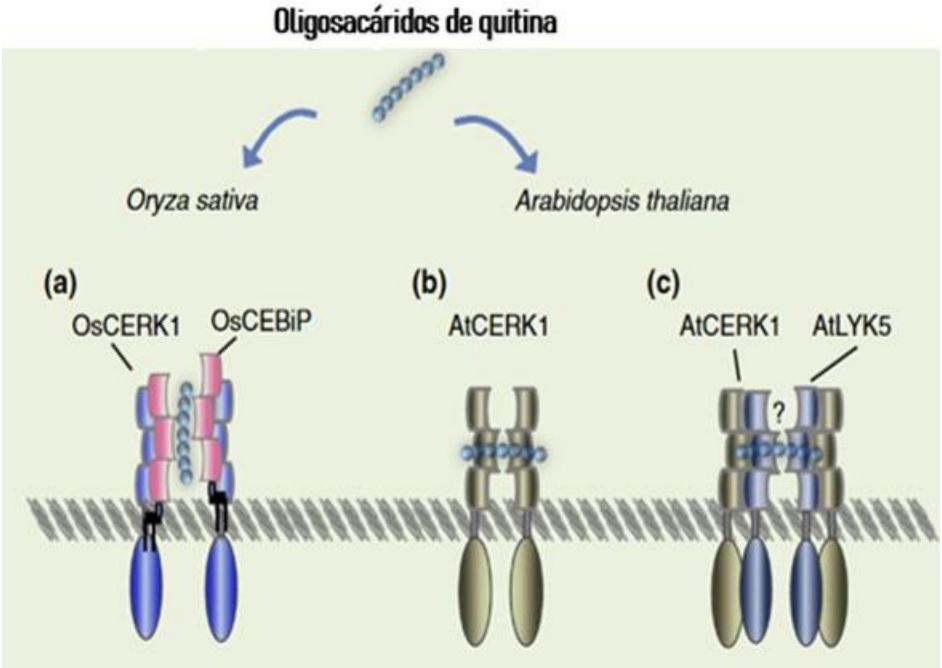

Figura 6. Esquema de unión de receptores RLP y RLK en planta de arroz y *Arabidopsis thaliana* (Tomado de la fuente Shinya *et al*., 2015).

Los receptores tipo cinasas (RLK) corresponden al patrón de receptores de reconocimiento de plantas (PRR, por sus siglas en inglés) localizados en la transmembrana que poseen dominios

extracelulares que son esenciales para transmitir información de estímulos externos al reconocer los ligandos (Beck *et al*., 2012; Monaghan y Zipfel, 2012; Muthamilarasan y Prasad, 2013). La transmisión de señales de defensa contra patógenos mediada por RLK responde al tratamiento con inductores, patógenos y moléculas de señalización producidas durante las respuestas bióticas. Las respuestas de las RLK a menudo dependen de ligandos y patógenos específicos (Malik *et al*., 2020). Por otro lado, en *Arabidopsis* se encontró una familia de proteínas relacionadas con la percepción de quitina, donde el receptor AtCERK1 es el más importante dentro de la familia de receptores RLK. Miya *et al*., (2007) afirmaron que el receptor de quitina tipo cinasa LysM-RLK en *A. thaliana* (AtCERK1) se une a fragmentos de quitina N-acetilada con tres motivos LysM y, a través de la formación de homodímeros, media las defensas inducidas por quitina. Wan *et al*., (2008) encontraron que AtCERK1 es el receptor primario de quitina (Iizasa *et al*., 2010; Petutschnig *et al*., 2010). Estudios previos sugirieron que los tres dominios LysM en el ectodominio de AtCERK1 son necesarios para la actividad de unión de quitina al formar un conjunto en forma de hoja de trébol (Petutschnig *et al*., 2010; Liu *et al*., 2012). El segundo dominio LysM (LysM2) proporciona una región de unión para $(GlcNAc)_5$ (Liu *et al*., 2012).

En el estudio realizado por Petutsching *et al*., (2010), los autores hicieron importantes contribuciones para comprender la función del receptor CERK1 en *Arabidopsis*. En ese trabajo se confirmó que en ausencia de CERK1, no se induce respuesta de defensa en presencia de quitina y que CERK1 resultó ser el ligando con mayor afinidad por la quitina. De la misma forma, el autor señaló la importancia de los dominios de lisina y destacó la importancia de la fosforilación de CERK1 tanto en el ectodominio para el proceso de reconocimiento de oligosacáridos de quitina a realizar, como en el dominio intracelular, para la transducción de la señal en la cascada de señalización. Posteriormente, Liu *et al*., (2012b) describieron la estructura cristalina de rayos X del ectodominio AtCERK1 y predijeron la interacción de los oligómeros de quitina con el segundo motivo LysM en el dominio extracelular. Estos autores sugirieron un modelo mediante el cual los oligómeros de quitina de cadena larga ($DP \geq 6$) se unen a los dominios LysM en dos monómeros, lo que da como resultado la homodimerización de AtCERK1. Se demostró que esta dimerización activa el dominio cinasa intracelular (Liu *et al*., 2012; Petutschnig *et al*., 2010).

En planta de arroz, dos receptores están involucrados en la inmunidad activada por quitina (Shimizu *et al*. 2010). La proteína de unión del inductor de quitina LysM-RLP (OsCEBiP) se une a fragmentos de quitina N-acetilados, lo que inicia la homodimerización del receptor y una mayor

heterodimerización con OsCERK1. Esta formación de heterotetrámeros desencadena la PTI inducida por quitina (Hayafune *et al*. 2014).

En *Arabidopsis* se han encontrado que existen cinco miembros de la familia de proteínas tipo cinasas similares al receptor con dominio de lisina (LYK), es decir, AtCERK1/LysM RLK1/AtLYK1 y AtLYK2-5 (Wan *et al*., 2012). Sin embargo, existe la posibilidad de que, de forma similar al arroz, el receptor de quitina activo esté compuesto por más de una proteína (Gubaeva *et al*., 2018).

Además de las proteínas receptoras de quitina, en *Arabidopsis* se han reportado miembros de la familia de cinasas similares al receptor con dominio de lisina (LYK). Las proteínas LysM de la planta funcionan principalmente como receptores de reconocimiento de patrones (PRR) que detectan la quitina para inducir la inmunidad de la planta (Hu et al., 2021). Cao y col. (2014) identificaron un receptor LysM-RLK en *Arabidopsis*, AtLYK5, que se une a la quitina con una afinidad más alta que AtCERK1. Los autores proponen que AtLYK5 funciona como el principal receptor de quitina, que recluta a AtCERK1 para formar un complejo de receptor inducible por quitina (Volk, 2019). Posteriormente, Erwig *et al*., (2017) comprobaron que CERK1 es esencial para el reconocimiento de los oligosacáridos de quitina en *Arabidopsis* y que existen otras proteínas receptoras que son importantes para que esta interacción sea efectiva. Específicamente LYK5, mostró ser requerida para los procesos de fosforilación y dimerización del receptor CERK1. Los autores concluyen que tanto CERK1, como LYK4 y LYK5 son proteínas inducibles y su presencia en membrana citoplasmática obedece a la presencia de quitina.

1.2.5.3 Reconocimiento de quitina y sus oligosacáridos en cultivos hortícolas. El reconocimiento de quitina y la relación entre los componentes del receptor se han estudiado principalmente en sistemas de plantas modelos como en arroz y *Arabidopsis*. Se han realizado pocos estudios para analizar la presencia de receptores de quitina y el modo de acción en la activación de las defensas en cultivos hortícolas. Zhang *et al*., (2019) hicieron importantes contribuciones para comprender la función biológica de un receptor de quitina en plátano (*Musa acuminata*). Para ello, los autores generaron plantas de plátano transgénicas silenciadas con ARN de interferencia (ARNi) para analizar el papel biológico de MaLYK1. Esta proteína es un receptor de quitina con dominio LysM localizada en la membrana plasmática, y pertenece a un subclado representado por AtCERK1

(AtLYK1) y OsCERK1 (OsLYK9). Se encontró que la expresión de MaLYK1 era mayor en las raíces de plátano, mientras que era menor en la fruta amarilla. Además, la expresión de MaLYK1 fue inducida por *Fusarium oxysporum* f. sp. *cubense* raza 4 (Foc4), y su función fue fundamental, no solo en el reconocimiento del hongo sino también en el reconocimiento del hongo en la relación simbiótica que se establece entre plantas y micorrizas. Además, el tratamiento con quitina $(GlcNAc)_8$ indujo la expresión de genes de defensa, como la fenilalanina amonio-liasa, la β-1,3-glucanasa y la proteína 1 relacionada con la patogénesis. En cambio, las líneas de plátano silenciadas con MaLYK1 mostraron una reducción de las respuestas de defensa activadas por la quitina y un aumento de los síntomas de la enfermedad inducida por Foc4. Los autores sugieren que el dominio cinasa de MaLYK1 podría reemplazar funcionalmente al del receptor cinasa 1 del inductor de quitina (*AtCERK1*) en *Arabidopsis*. MaLYK1 representa un interruptor molecular central que controla la señalización relacionada con la defensa y la simbiosis.

Estudios similares reportan la presencia del receptor MdCERK1 en manzana (*Malus domestica*), en presencia del hongo *Rhizoctonia solani*, observándose un aumento en la expresión de *MdCERK1* en raíces y hojas. Se identificó el gen *Md09g1111800* el cual se sugiere que codifica a una proteína tipo cinasa similar al receptor de unión a quitina, basándose en la similitud de 18 secuencias con *AtCERK1* de *Arabidopsis*. Esta proteína se encontró en la membrana plasmática y tiene tres dominios ricos en lisina en su zona extracelular, un dominio transmembrana y un dominio intracelular con motivos serina / treonina. Se concluyó en dicho estudio que el gen *Md09g1111800* es un ortólogo de *AtCERK1* y que participa en la percepción de quitina (Zhou *et al*., 2018). Recientemente, se identificó otro gen que codifica al receptor tipo CERK, designado como *MdCERK1–2* en manzanas. Este gen codifica a una proteína con alta similitud con MdCERK1 y AtCERK1 previamente reportados. Los autores reportaron que la expresión de *MdCERK1-2* en la manzana fue inducida por *Botryosphaeria dothidea* y *Glomerella cingulate*. Además, se observó que la sobreexpresión de *MdCERK1-2* en plantas transgénicas de *Nicotiana benthamiana* mejoró su resistencia a la infección por *A. alternata*. Estos resultados sugieren que *MdCERK1-2* está involucrado en las respuestas de defensa de la manzana contra hongos patógenos (Chen *et al*., 2020).

En tomate (*Solanum lycopersicum*), Zheng *et al*., (2012) analizaron las proteínas de la planta de tomate que interactúan con AvrPtoB con el fin de investigar los mecanismos de virulencia de AvrPtoB. Los autores identificaron a Bti9, una proteína tipo cinasa similar al receptor CERK1 con

dominios de LysM. Bti9 tiene una elevada similitud de aminoácidos con el receptor CERK1 en *Arabidopsis* y pertenece a un clado que contiene otras tres proteínas de tomate, SlLYK11, SlLYK12, y SlLYK13, las cuales interactúan con AvrPtoB. Los autores sugieren que estas proteínas están involucradas en la respuesta a PAMPs que activan la cascada de señalización.

Liao *et al*., (2018), analizaron la expresión de los genes *SlLYK1*, *SlLYK12* y *SlLYK13* después de aplicar un tratamiento con ChO_8 en hojas y raíces de tomate, con el objetivo de demostrar que eran requeridos para la señalización de quitina. La expresión del gen *SlLYK1* se incrementó siete veces después del tratamiento con ChO_8 en hojas y 3 veces en raíces; mientras que *SILYK12* se expresó en respuesta a la relación simbiótica con micorrizas. *SlLYK13* también incrementa ligeramente su expresión en las hojas después del tratamiento con ChO_8, pero se observó una mayor sobreexpresión relacionada con la muerte celular programada. Estos resultados sugieren que los genes que codifican a receptores de quitina tienen distintas funciones en la planta dependiendo del tejido o del PAMP aplicado. Los autores construyeron un árbol filogenético analizando las secuencias de proteínas homólogas a CERK1 de seis especies de *Leguminosae*, tres especies de *Solanaceae*, tres especies de *Cruciferae* y dos especies de *Gramineae*. Utilizaron secuencias de aminoácidos de longitud completa codificadas por 48 genes de 14 especies y asignaron los homólogos CERK1 de monocotiledóneas y las especies dicotiledóneas a dos grupos diferentes de manera similar a otros árboles filogenéticos de proteínas o cinasas receptoras LysM previamente reportados (Arrighi *et al*., 2006; Zhang *et al*., 2007; Lohmann *et al*., 2010; De Mita *et al*., 2014). Los autores encontraron cuatro ortólogos de CERK1 en tomate (SlLYK1, SlLYK11, SlLYK12 y SlLYK13) e investigaron sus roles en la señalización de quitina y simbiosis con micorrizas arbusculares (AM). Encontraron que la eliminación de SlLYK12 en tomate redujo significativamente la colonización por AM, mientras que las respuestas inducidas por quitina no se vieron afectadas. Por el contrario, la eliminación de SlLYK1 dio como resultado respuestas reducidas a la quitina, pero no alteró las respuestas a AM (Liao *et al*., 2018). Lo anterior sugiere que existen en tomate genes homólogos al gen CERK1 involucrado en la percepción de quitina.

Por otra parte, una vez que los oligosacáridos de quitina son reconocidos por los receptores de membrana, la señal se transduce al interior de la célula a través de proteínas citoplasmáticas que se unen al dominio intracelular del receptor, activando la cascada de señalización y la activación de la respuesta inmune (Gubaeva *et al*., 2018). En ese sentido, AbuQamar *et al*., (2008) y Ray *et al*., (2015) identificaron el comportamiento del gen *TPK1b* (Tomato protein kinase 1b) en la respuesta

de defensa en planta de tomate frente a *Botrytis cinerea y Alternaria solani,* respectivamente. Los autores identificaron la influencia del gen *TPK1b* en la respuesta de defensa frente a hongos necrótrofos y demostraron que este gen codifica para un receptor tipo cinasa y es ortólogo de la proteína cinasa CERK1 presente en *Arabidopsis,* por lo que posiblemente esté involucrado en el reconocimiento de quitina. Se demostró que en la respuesta de defensa mediada por la sistemina, TPK1b fue fosforilado por PORK1 (PEPR1/2 ortholog receptor-like kinase 1; PEPR1/2, recognize *Arabidopsis* peptides), el cual es ortólogo de RLK1. En el mismo estudio, cuando se silenció PORK1, los niveles de fosforilación de TPK1b disminuyeron, lo que afectó a la respuesta de defensa a través de esta vía (Xu *et al*., 2018). Estos estudios reafirman que TPK1b tiene un papel esencial en la transducción de la señal del receptor RLK en diversas respuestas de defensa de las plantas.

A pesar de existir en la literatura estudios sobre receptores que reconocen oligosacáridos de quitina en diferentes modelos de plantas como arroz y *Arabidopsis*, los estudios en planta y fruto de tomate aún son escasos. Asimismo, se desconocen los genes que codifican para estos receptores involucrados en el reconocimiento de oligosacáridos de quitina de *A. alternata* en fruto de tomate.

1.3 Hipótesis

Existen genes que codifican para receptores de membrana en fruto de tomate, que se expresan en respuesta a la aplicación de fragmentos de quitina de *Alternaria alternata,* induciendo la síntesis de las moléculas de señalización, y activando el mecanismo de defensa.

1.4 Objetivo General

Evaluar los genes que codifican para receptores de membrana en frutos de tomate expuestos a los fragmentos de quitina de *Alternaria alternata,* así como su nivel de expresión.

1.5 Objetivos Específicos

1. Obtener y caracterizar parcialmente los oligosacáridos de quitina de *A. alternata.*
2. Determinar el efecto inductor de los fragmentos de quitina de *A. alternata* en fruto de tomate, a través de la actividad de la quitinasa.
3. Establecer el (los) tiempo(s) en el (los) que ocurre mayor nivel de expresión de los genes posiblemente involucrados en el reconocimiento de quitina fúngica.
4. Identificar mediante RNA Seq los genes inducidos que codifican para receptores de membrana en los frutos de tomate expuestos a fragmentos de quitina de *A. alternata.*

1.6 Sección Integradora del Trabajo

La presente tesis está integrada por 4 artículos científicos relacionados con los objetivos de la investigación desarrollada, un artículo de revisión y 3 artículos de investigación que se presentan en los capítulos 2, 3, 4 y 5. En el capítulo 2 se presenta un artículo de revisión que incluye una extensa revisión bibliográfica del estado del arte sobre el reconocimiento de quitina en diferentes modelos de plantas y frutos, así como la influencia de esta molécula en la activación de las rutas de señalización y del mecanismo de defensa natural. En este capítulo se abarcan espectos sobre los problemas postcosecha en fruto de tomate, así como los eventos moleculares que ocurren durante la interacción planta-patógeno. Se describen los receptores de membrana tipo RLK y RLP que participan en la interacción planta-patógeno los cuales reconocen a los oligosacáridos de quitina, así como el efecto de ciertas propiedades de esas moléculas en el proceso de reconocimiento por dichos receptores. Además, se describen los mecanismos moleculares de reconocimiento de quitina tanto en plantas de *Arabidopsis* y arroz, como en ciertos cultivos hortícolas.

El tercer artículo comprende un artículo de investigación en el cual se realizó una comparación sobre dos métodos de obtención de quitina, el químico y el enzimático. El método químico ha sido ampliamente reportado en la obtención de quitina de origen marino y consta de una hidrólisis alcalina con hidróxido de sodio a altas temperaturas. Se aplicó este tratamiento al hongo necrótrofo

A. alternata con el fin de extraer la quitina presente en su pared celular. Sin embargo, este proceso es muy agresivo, provocando la desacetilación de la molécula de quitina (al visualizar las bandas de los enlaces en el Espectro Infrarrojo con Transformada de Fourier) y la obtención de un material rígido, insoluble y de difícil manejo.

En la literatura se ha reportado que los oligosacáridos de quitina son solubles en agua, al ser extraídos con el uso de enzimas, obteniéndose productos más estables químicamente y más activos biológicamente que la quitina. Debido a esto se utilizó el método de obtención enzimático, a través de proteasas y glucanasas comerciales, las cuales provocaron la ruptura de los enlaces de los componentes presentes en la pared celular del hongo, permitiendo la extracción de la quitina. Posteriormente se aplicó la sonicación, un proceso mecánico que favoreció la obtención de fragmentos de quitina. Estos fragmentos se caracterizaron de acuerdo a sus propiedades físicas y químicas. Se concluyó que el método enzimático fue más efectivo para obtener fragmentos de quitina de origen fúngico altamente acetilados y parcialmente solubles.

El cuarto capítulo comprende un segundo artículo de investigación en el cual se determinó el efecto inductor de los fragmentos de quitina de *A. alternata*, sobre el mecanismo de defensa en frutos de tomate. En este artículo se describe la aplicación de fragmentos de quitina de bajo peso molecular de *A. alternata* sobre el fruto de tomate en estado de madurez rosa, evaluando el efecto del tratamiento durante varios tiempos posteriores a la exposición. Se determinó la actividad de la enzima quitinasa (proteína relacionada a la patogénesis involucrada en la degradación de la quitina) así como el nivel de expresión del gen *Chi1* que codifica para esa proteína. Se observó que los fragmentos de quitina indujeron un aumento significativo en la actividad de la quitinasa, así como una sobreexpresión significativa del gen *Chi*1 a los 30 min posteriores a la aplicación. Se concluyó que el fruto de tomate responde casi de manera inmediata a la aplicación de los fragmentos de quitina y que estos inducen el mecanismo de defensa en el fruto.

El quinto capítulo se trata de un tercer artículo de investigación en el cual se identificaron mediante RNA-Seq los genes homólogos/ortólogos que posiblemente están involucrados en el reconocimiento de fragmentos de quitina de *A. alternata*, así como su participación en la activación de las rutas de señalización y mecanismo de defensa en frutos de tomate. Para ello se crearon de 6 genotecas a partir de RNAt, generándose 20 millones de lecturas. Se mapearon las secuencias limpias en el genoma del tomate utilizando la plataforma del Sol Genomics y se realizó el análisis *in silico* de la expresión diferencial. Se validaron los resultados obtenidos *in silico* mediante PCR

en tiempo real, obteniendo una alta correlación entre ambos métodos. Se comprobó que en tomate existen genes que codifican para receptores de quitina tipo cinasas, los cuales son homólogos a los reportados en planta de tomate, así como genes ortólogos a CERK1 presente en *Arabidopsis*, que están involucrados en el reconocimiento de quitina.

2. TOWARD UNDERSTANDING THE MOLECULAR CHITIN RECOGNITION AND ACTIVATION OF THE PLANT DEFENSE MECHANISM IN HORTICULTURAL CROPS

Artículo en revisión por la revista Molecules. Enviado

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Abstract

Large volumes of fruit and vegetable production are lost during postharvest handling due to attacks by necrotrophic fungi. One of the promising alternatives proposed for the control of postharvest diseases is the induction of natural defense responses that can be activated by recognizing molecules present in pathogens, such as chitin. Chitin is one of the most important components of the fungal cell wall that is recognized through plant membrane receptors. These receptors belong to the receptor-like kinase (RLK) family, which possesses a transmembrane domain and/or receptor-like protein (RLP) that requires binding to another RLK receptor to recognize chitin. In addition, these receptors have extracellular LysM motifs that participate in the perception of chitin oligosaccharides. These receptors have been widely studied in *Arabidopsis thaliana* and *Oryza sativa*; however, it is not clear how chitin oligosaccharides' molecular recognition and plant defense mechanisms occur in other plant species or fruits. This review includes recent findings on the molecular recognition of chitin oligosaccharides and how chitin oligosaccharides activate defense mechanisms in plants. In addition, we highlight some of the current advances in chitin perception in horticultural crops.

Keywords: chitin oligosaccharides, chitin elicitor receptors, plant immunity, horticultural crops

1. Introduction

One of the most important problems for the horticultural industry that has a negative impact on food security is the postharvest decay of fruits and vegetables [1]. Fruits and vegetables are highly susceptible to decay caused by necrotrophic fungi such as *Alternaria alternata*, *Botrytis cinerea*, and *Colletotrichum gloeosporioides*, among others, even under low temperature storage conditions [2,3]. The primary means to control fungal diseases is the use of synthetic fungicides; however, their use has caused concern due to their possible negative effects on human health, their toxic residuality, their long degradation period and the induction of resistant strains [4]. In this sense, safer alternatives to control postharvest diseases and guarantee food safety are needed. A promising alternative is the induction of natural plant defense mechanisms, which are a complex network of biochemical and molecular events that can limit the penetration and invasion of pathogens in plant tissue, preventing or decreasing disease development [5,6].

The plant defense mechanism is activated once the spore of a pathogen comes in contact with the plant surface. Furthermore, pathogen-associated molecular patterns (PAMPs) are recognized by plant membrane receptors, known as pattern recognition receptors (PRRs), and activate signal transduction to the nucleus [7]. Chitin is present in the fungal cell wall, and it has been shown to participate in plant-pathogen recognition phenomena [8,9] and in the activation of innate plant defense mechanisms [10]. The presence of receptors in plants that recognize chitin and chitin oligosaccharides (ChOs) has been widely demonstrated. Furthermore, it has been reported that the recognition of these ChOs and the activation of signal transduction into the plant cell are more effective depending on the receptor type and certain physicochemical characteristics of the ChOs, such as molecular size, number of units, and acetylation degree [11-14].

Currently, two families of chitin receptors have been reported in plants, receptor-like kinases (RLKs) and receptor-like proteins (RLPs), both of which are fundamental components for detecting MAMPs or PAMPs [15]. These membrane receptors that recognize chitin have been widely studied in *Oryza sativa* (rice) and *Arabidopsis thaliana*. Chitin elicitor-binding protein receptors such as CEBiP were the first chitin RLPs found in rice and are characterized by extracellular lysine domains that interact with an RLK for chitin recognition. CEBiP contains two extracellular LysM motifs and one transmembrane domain; however, it does not possess intracellular domains to initiate signal transduction; hence, it requires additional proteins [16,17]. On the other hand, RLKs, such as chitin elicitor receptor kinase (CERK1), are one of the main chitin receptors found in *Arabidopsis*. They include extracellular Lys domains that are probably involved in the interaction with PAMPs, a transmembrane domain and a cytoplasmic kinase domain with basic auto phosphorylation/myelin protein (MBP) kinase activity, which can initiate a signaling cascade within the cell [18,19]. In addition to chitin receptor proteins, some studies have reported that there are other receptor proteins that are members of the lysine domain receptor-like kinase (LYK) family in *Arabidopsis* which plays a role in the interaction with PAMPs [20]. When ChOs are recognized by PRRs, the signaling pathways starts and a complex defense response is activated, including the transcription of defense genes in the nucleus [21].

The recognition of ChOs by plant receptors has been reported mainly in *Arabidopsis* and rice; conversely, there are scarce studies in horticultural crops. According to some authors [22,23], ChOs are multicomponent transmembrane complexes that are related but differ in detail between species. Therefore, it is not clear how the molecular recognition of ChOs and the activation of downstream

signaling occur in horticultural crops. The main objective of this review is to present an overview of recent findings regarding the molecular recognition of chitin oligosaccharides and how they activate defense mechanisms in plants. In addition, we highlight some of the current advances about how this phenomenon of recognition occurs in horticultural crops.

2. Molecular mechanism of plant response to fungal pathogens

Over the course of evolution, plants have developed different strategies to resist and protect themselves from pathogen attacks. The first defense barrier in plants is the physical or structural barrier (waxes, cuticle, trichomes, etc.) that prevents pathogens and predators from invading the plant cell [23]. When plants become infected, several changes in the cuticle take place, which causes the activation of defense responses [24]. Once pathogens overcome the first line of defense, the second line of defense is initiated based on the cellular innate immunity, which allows the plant to resist or block the pathogen [25]. Among the main components that participate in this immune system are the PRRs found in the plasma membrane of plant cells, which recognize highly conserved molecular patterns called microbial-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs). These are products secreted by microorganisms or released from their cell walls by hydrolytic enzymes during plant-pathogen interactions. MAMPs include peptidoglycans from gram-positive bacteria, lipopolysaccharides from gram-negative bacteria, glucans, proteins, or flagellin [26], while chitin and its oligosaccharides are considered PAMPs [27]. The perception of these molecular patterns during infection by pathogens triggers defense reactions known as PAMP-triggered immunity (PTI). In this regard, chitin and its ChOs are considered general elicitor compounds capable of inducing a defense response in plant cells [28,29].

When MAMPs or PAMPs are recognized by PRRs, the defense responses are induced through the signaling cascade by mitogen-activated protein kinases (MAPKs) and calcium –dependent protein kinase (CDPK). The message induces the production of signaling hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), auxins (AUX), cytokinin (CK), gibberellins (GB), and brassinosteroids (BR) [6,30,31]. Through these signaling pathways, a complex defense response is activated, including modifications to create structural defenses (random creation of bonds between cell wall polymers, lignification), the induction of reactive oxygen species (ROS), and nitric oxide (NO) (Figure 1). This activation has a role in transcriptional

reprogramming and induction of high expression of early defense-related genes [32,33]. These genes encode pathogenesis-related (PR) proteins such as glucanases, chitinases, peroxidases, and enzymes such as phenylalanine ammonia-lyase, a key enzyme involved in the synthesis of phytoalexins [34,35], which play important roles in the defense against pathogens [6,36-38]. In this context, a successful plant defense mechanism should inhibit pathogen infection and protect the plants against future attacks.

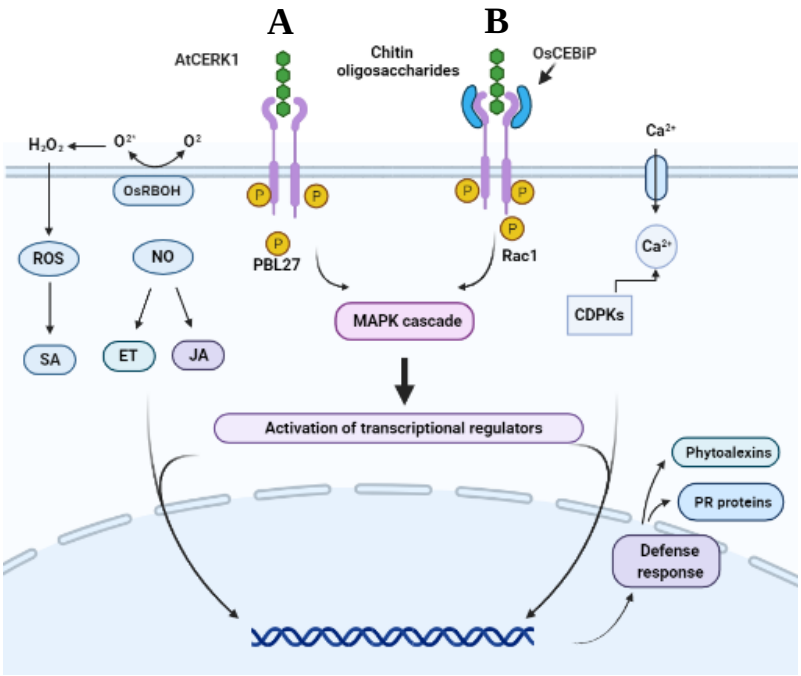

Figure 1. Representative scheme of chitin oligosaccharide perception by the OsCEBiP/OsCERK1 dimer. Structural differences between the RLP (OsCEBiP) and RLK (OsCERK1) receptors are shown. (In Figure 1A, chitin oligosaccharides bind to CERK1 in *Arabidopsis.* This complex sends the signal to the intermediate protein, which will begin to phosphorylate and trigger the MAP kinase pathway, induces the accumulation of SA, JA, ET, NO, and ROS, activating the defense responses. In Figure 1B, the CEBiP receptor in rice binds to CERK1, forming a dimer complex that will allow it to recognize chitin oligosaccharides and activate its defense mechanism in the same way). Figure created using BioRender (https://biorender.com/).

3. **Cellular recognition of fungal molecules: chitin**

The fungal cell wall is made up of glycoproteins, glucans and chitin, among other molecules. There is a growing interest in chitin since it has been shown that this molecule is essential in plant-pathogen recognition [9], and because it can induce the activation of the innate plant defense mechanism [10]. Chitin molecules are composed of sugars linked by glycosidic bonds β (1 → 4), forming a linear chain of N-acetyl-2-amino-2-deoxy-D-glucose units, which are the second most abundant polymers in nature after cellulose [39,40]. Microscopically, it is in crystalline or semi-crystalline form [41], making this polysaccharide a rigid and resistant material that acts as a very powerful barrier in the fungal cell wall and protects the organisms themselves [10]. Chitin is an insoluble polysaccharide when extracted by alkaline treatment and partially soluble when extracted by enzymatic treatment, from which other compounds of high biological significance are derived, such as chitosan and ChOs [42]. According to Buendia *et al.* [17] and Roy *et al.* [43], ChOs are soluble depending on the pH and degree of polymerization (dp).

At the level of molecular recognition, some studies indicate the relationship of chitin and ChOs with the induction of plant defense mechanisms. Recent studies relate chitin as an enhancer of the biological control by *Rhodotorula mucilaginosa* yeast against *Rhizopus* infection in peach crops. The addition of 0.5% of chitin reduced the postharvest disease of fruits by blue mold up to half. In these fruits, differential expression of proteins was observed concerning the untreated fruits, including glucosidases, hydrolases, NADH dehydrogenases, and kinases with serine/threonine domains, which are decisive in the plant response defense against the pathogen [44]. Similarly, the addition of chitin isolated from the yeast cell wall (*Saccharomyces cerevisiae*) in tomato fruit was correlated with increased expression levels of enzymes of oxidative metabolisms, such as the enzymes superoxide dismutase, peroxidase, and catalase, as well as enzymes inherent to the response to fungal infection, such as glucanase and chitinase [45].

4. Plant membrane receptors that recognize chitin and ChOs

Currently, two families of chitin receptors are reported in plants, RLKs and RLPs, which are fundamental components for detecting MAMPs or PAMPs [15,46]. These are related multicomponent transmembrane complexes, but they showed differences between species [22].

RLKs are a group of membrane proteins with an extracellular region including a sensor domain, a transmembrane region, and an intracellular region with a Ser/Thr kinase domain with homology to protein kinases involved in signal transduction. Furthermore, they are divided into subfamilies

according to the amount of lysine residues in their extracellular region [47]. By other side, RLP receptors lack an intracellular domain, and in some cases are anchored to the membrane through a transmembrane domain of another protein, or a glycosyl-phosphatidyl inositol group [17,29,48]. These membrane receptors of the RLK or RLP types have been widely studied and characterized in rice and *Arabidopsis*. In rice plants, chitin elicitor-binding protein (CEBiP) which recognizes the N-acetyl groups of chitin by the lysine domains (LysM) of the receptor [21,49], was the first chitin receptor identified [16]. The molecular weight of this protein was estimated at 75 kDa, and it has 11 glycosylation sites through which N-acetyl glucosamine (GlcNAc) can bind. According to some authors, in rice plants, two receptors are involved in chitin-activated immunity [50]. LysM-RLP (OsCEBiP) binds to N-acetylated chitin fragments, initiating receptor homodimerization and further heterodimerization with OsCERK1, initiating PTI [51]. Additionally, the authors showed that in the OsCEBiP receptor, the central lysine motif is essential for recognizing ChOs with a dp of 8. CEBiP is dimerized with the OsCERK1 protein for binding to these oligosaccharides (Figure 1). In this sense, the chitin receptor system in rice requires both CEBiP and OsCERK1 for chitin sensing and signaling.

In *Arabidopsis*, a family of proteins related to chitin perception was found, and the CERK1 receptor is the most important within the RLK receptor family. LysM-RLK in *Arabidopsis* (AtCERK1) binds to N-acetylated chitin fragments with three LysM motifs and mediates the plant defenses induced by chitin through the formation of homodimers [12,18]. Important findings to understand the function of the CERK1 receptor in *Arabidopsis* were made by Petutsching *et al.* [13]. It was confirmed that in the absence of CERK1, no defense response was induced in *Arabidopsis* exposed to chitin, and CERK1 was the ligand with the highest affinity for chitin. Additionally, the authors highlighted the importance of the lysine domains and the phosphorylation of CERK1 in the ectodomain for the recognition process of ChOs and the intracellular domain to initiate signal transduction.

According to some studies, the recognition of ChOs is more effective depending on their polymerization and acetylation degrees. For instance, in the cork tree *Hevea brasilien*sis, a linear relationship of 1:1 for $(GlcNac)_5$ with the receptor was shown, and for $(GlcNac)_{8,}$ the ratio was (2:1) protein-oligosaccharide, indicating dimerization of receptors for the recognition of ChOs with higher dp [11], while $(GlcNac)_{6\text{-}8}$ is recognized by membrane receptors in *Arabidopsis* [52]. In

another study, $(GlcNac)_{5-9}$ was recognized with great efficiency by the CERK1 receptor of *Arabidopsis* [12], while ChOs with a lower dp did not show affinity for the CERK1 receptor [13]. Later, Liu *et* al. [53] described the X-ray crystal structure of the AtCERK1 ectodomain and predicted the interaction of ChOs with the second LysM motif of the extracellular domain. These authors suggested that long chain chitin oligomers (dp ≥ 6) bind to LysM domains in two monomers, resulting in the homodimerization of AtCERK1. This dimerization was shown to activate the intracellular kinase domain [13,53]. However, there is a possibility that, as in rice, the active chitin receptor in *Arabidopsis* can be a complex of more than one protein [22].

In addition to chitin receptor proteins, members of the lysine domain receptor-like kinase (LYK) family have been reported in *Arabidopsis*. The plant LysM proteins mostly function as pattern recognition receptors (PRRs) that recognizes chitin to induce the plant's immunity [26]. Cao *et al.* [20] identified a LysM-RLK in *Arabidopsis* (AtLYK5) that binds to chitin with a higher affinity than AtCERK1. The authors propose that AtLYK5 functions as the major chitin receptor, recruiting AtCERK1 to form a chitin-inducible receptor complex, which agrees with the results reported by other authors [54]. Erwig *et al.* [55] demonstrated that CERK1 is essential for recognizing ChOs in *Arabidopsis* and reported other receptor proteins involved in chitin perception. Specifically, LYK5 was shown to be required for the phosphorylation and dimerization processes of the CERK1 receptor. The authors concluded that CERK1, LYK4 and LYK5 were inducible proteins and that their presence in the cytoplasmic membrane was due to the presence of chitin and depended on a complex network of vesicular trafficking. Therefore, there are proteins that bind the intracellular domains of receptors and activate the signaling cascade, and kinase activity is required to activate immune responses [22,53]. For example, PBL27 is the downstream component of AtCERK1 after chitin perception, and it can be phosphorylated by AtCERK1 [56]. PBL19 and PBL27 phosphorylate MAPKKK to activate MAPK cascades, which in turn activates defense genes in the nucleus [57]. In summary, the receptors for these chitin patterns, such as CEBiP/CERK1, are activated by ligand binding and trigger various immune responses.

5. **Recognition of chitin and its oligosaccharides in horticultural crops**

The chitin receptor complex and the relationship among the components have been mostly studied in rice and Arabidopsis model systems. However, few studies have been performed to study the presence of chitin receptors and mode of action in defense activation in horticultural crops. For

instance, Zhang *et al*. [58] made important contributions to understanding the biological function of a chitin receptor-binding protein in banana (*Musa acuminata*). The authors confirmed the presence of the MaLYK1 protein in the plasma membrane. This protein is a LysM-domain-chitin receptor that belongs to a subclade represented by AtCERK1 (AtLYK1) and OsCERK1 (OsLYK9). The expression of MaLYK1 was found to be higher in banana roots, while it was lower in yellow fruit. In addition, MaLYK1 expression was induced by *Fusarium oxysporum* f. sp. *cubense* race 4 (Foc4), and its function was essential, not only in recognizing the fungus but also in recognizing the fungus in the symbiotic relationship established between plants and mycorrhizae. Besides, chitin treatment $(GlcNAc)_8$ induced the expression of defense genes, such as phenylalanine ammonia-lyase, β-1,3-glucanase, and pathogenesis-related protein 1. Similar studies reported the activation of the MdCERK1 receptor in apple (*Malus domestica*) in the presence of *Rhizoctonia solani* infection, observing that MdCERK1 expression increased in roots and leaves. This protein was found in the plasma membrane and has three lysine-rich domains in its extracellular zone, a transmembrane domain, and an intracellular domain with serine/threonine motifs [59]. Recently, another CERK gene, designated as MdCERK1–2, was identified in shoot barks of apples. It encodes a protein with high similarity with the previously reported MdCERK1 and AtCERK1. Results revealed that MdCERK1–2 expression in apple was induced by *Botryosphaeria dothidea* and *Glomerella cingulate*. Additionally, it was observed that the MdCERK1–2 overexpression in transgenic *Nicotiana benthamiana* plants improved their resistance to *A. alternata* infection. These results suggest that MdCERK1–2 is involved in apple defense responses against pathogenic fungi [60].

In tomato (*Solanum lycopersicum*), Bti9 interacts with the AvrPtoB protein from *Pseudomonas syringae* [61]. Bti9 is a protein kinase with 76% similarity with LysM CERK1 found in *Arabidopsis,* which shares a clade with SlLYK11, SlLYK12, and SlLYK13. The authors suggested that these proteins are involved in the response to PAMPs that trigger the signaling cascade. Liao *et al*. [62] showed the relationship between homologous CERK1 receptors in tomato (SlLYK1, SlLYK12 and SlLYK13) and colonization of arbuscular mycorrhizae (AMs). The authors found that *SlLYK1* gene expression was upregulated after chitin oligosaccharide treatment, whereas *SlLYK12* was involved in AM symbiosis. Another interesting piece of data to elucidate the phenomena of chitin recognition showed that cell death was induced by overexpressing SlLYK13, suggesting that those chitin receptor genes have distinct functions in plants depending on the

specific plant tissue or PAMPs applied. As well as in *Arabidopsis*, when ChOs are perceived in tomato, there are proteins that bind the intracellular domains of the chitin receptor and activate the signaling cascade which include kinase activity that is required for the activation of immune responses [22]. In tomato, the TPK1b protein is probably an ortholog of BIK1 in *Arabidopsis*, which is essential in the defense response of *Arabidopsis* against *B. cinerea*. The susceptibility to *B. cinerea* was increased when TPK1b was inhibited by RNAi [63]. Another study found that in the defense response mediated by systemin, TPK1b was phosphorylated by the ortholog of RLK1 (PORK1). In the same study, when PORK1 was silenced, the levels of TPK1b phosphorylation decreased to the same extent, which affected the defense response through this pathway [64]. These studies further support the essential role of TPK1b in the transduction of the RLK receptor signal in various plant defense responses.

In summary, it was found that *Arabidopsis thaliana* and *Oryza sativa* have a group of receptors with high affinity for ChOs and that the interactions in this recognition is fairly well understand. In the same way, one of these receptors (RLK type) was described in certain horticultural crops in which the structural patterns are preserved. Additionally, the ChOs role in the activation of the defense mechanism was also observed in horticultural crops.

6. Concluding Remarks and Future Perspectives

Great advances have been achieved to elucidate the molecular recognition of chitin oligosaccharides by plant membrane receptors, and the functional receptor complex, based on studies performed in the model plants *Arabidopsis thaliana* and *Oryza sativa*.

According to the few studies reported in horticultural crops, it is possible to suggest that the molecular mechanism of chitin oligosaccharide perception occurs through RLK-type receptors with lysine domains, such as the CERK1 receptor reported in *A. thaliana*. Nevertheless, the details of the receptor complex formation induced by chitin oligosaccharides and the activation of signaling and defense responses within the cell are not clear. Therefore, future studies are required to fully elucidate the molecular mechanism of the response to pathogen attack.

Understanding the molecular mechanism of pathogen recognition and the activation of the defense mechanism would help with the problem of horticultural postharvest losses and food supply by

providing safe and effective alternatives for the development of disease-resistant crops and/or food preservation without using the synthetic chemical compounds.

7. References

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3. ENZYMATIC TREATMENTS AS ALTERNATIVE TO PRODUCE CHITIN FRAGMENTS OF LOW MOLECULAR WEIGHT FROM *Alternaria alternata*

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Journal of Applied Polymer Science

Enzymatic treatments as alternative to produce chitin fragments of low molecular weight from *Alternaria alternata*

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ABSTRACT: Chitin and its oligosaccharides are involved in the plant defense response against fungal pathogens. In most studies, these molecules come from crustacean shell, and there are scarce studies on the use of fungal chitin. Usually the extraction of chitin is by alkaline treatments, which affect the acetylation degree (DA), and the obtaining of oligosaccharides of low molecular weight; so the use of enzymes is proposed as an alternative treatment to obtain chitin oligosaccharides from fungi. The objective of this work was to characterize the chitin fragments of *Alternaria alternata* extracted by alkaline and enzymatic treatments. The chitin extracted was ultrasonicated and ultrafiltrated to produce chitin fragments. Enzymatic treatments decreased the protein content to 29%, and the bands corresponding to β-glucans decreased when β-1,3-glucanase was applied. Fragments smaller than 1 kDa, DA of 76.7%, and 35.4 μg glucosamine/mg dry weight were obtained by enzymatic treatments. The enzymatic treatments show promising results to extract chitin fragments from *A. alternata* without severely affect the DA of the molecule. © 2018 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2019**, *136*, 47339.

KEYWORDS: *Alternaria alternata*; FT-IR; fungal chitin fragments; glucanase treatments; protease treatments

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INTRODUCTION

Chitin is widely distributed in nature, in the animal, plant, and fungi kingdoms. It is the second most abundant natural polysaccharide surpassed only by cellulose, and is found mainly in the shells of crustaceans, the exoskeletons of insects, and the cell walls of fungi.[1] Structurally, chitin is a polysaccharide, insoluble in aqueous solution, which has a linear structure composed by repetitive units of *N*-acetyl-D-glucosamine (GlcNAc), linked by glycosidic bonds of type ß-(1 → 4).[2]

There is a growing interest in the study of chitin and its oligosaccharides, as it has been shown that both are involved in the induction of plant defense response to fungal pathogens.[3,4] Some studies reported that the recognition of chitin and its oligosaccharides occurs through plant membrane receptors. This interaction takes place through the lysine domains of the plasma membrane receptor with *N*-acetyl groups of chitin and is related to the polymerization degree and acetylation.[5]

Zhang *et al.*[6] showed that oligosaccharides with a polymerization degree between 6 and 8 ($r(GlcNac)_6$ and $r(GlcNac)_8$) were recognized by membrane receptors in *Arabidopsis thaliana*. Likewise, Iizasa *et al.*[7] found that chitin oligosaccharides with a polymerization degree of 5–9 ($r(GlcNac)_5$–$r(GlcNac)_9$ were recognized by the RLK1 receptor to a degree greater than 75%. Liu *et al.*[8] reported similar results for chitin oligosaccharides with a polymerization degree of 8 ($r(GlcNac)_8$. Most chitin and its oligosaccharides used in those studies are from crustaceans shell; however, no scientific information has been found about the plant recognition of chitin and its oligosaccharides from fungi like *Alternaria alternata*. This fungus is responsible for the black rot disease in a wide range of fruits and vegetables.[9] Currently, its control is based on chemical methods, but its use has been restricted due to possible negative effects on human health like toxic residuality, long period of degradation, and the induction of resistant strains.[10] Therefore, there is a growing public and

scientific interest in the search for ecologically safer alternatives that guarantee the safety of food, which can be done through the induction of natural defense mechanism. To get a better understanding of the recognition of fungal chitin in fruits, further studies are needed which will require partially deacetylated fungal chitin.

Chitin is usually extracted from the shells of crustaceans by chemical treatment. This treatment consists of a strong alkaline hydrolysis with sodium hydroxide at high concentrations and temperatures during several hours of treatment, which leads to the rupture of the chains producing a high deacetylation degree (DA) of the polymer.[1,11] This high degree of deacetylation of the molecule could avoid the recognition of the *N*-acetyl-glucosamine groups of the chitin oligosaccharides since it has been shown that it only occurs when it is partially acetylated.[7,12]

Most of these alkaline treatments have been used to get chitosan, a molecule obtained from the deacetylation of chitin extracted mainly from shrimp exoskeleton. In the case of fungal chitin, which is linked to glycoproteins and glucans, it requires other extraction methods without affecting drastically the DA of the molecule. Enzymatic treatments with proteases and glucanases have been reported that can eliminate proteins and glucans present in samples of the fungal cell wall, while the DA of the chitin molecule is retained.[13]

Younes *et al.*[13] recovered chitin through the enzymatic deproteinization of shrimp processing byproducts. They evaluated different microbial and fish viscera proteases to determine its deproteinization effectiveness and they recorded high levels of protein elimination. In fungi, Sivan and Chet[14] reported an enzymatic treatment based on the lytic enzyme β-1,3-glucanase produced from *Trichoderma harzianum* that degraded *Fusarium oxysporum* cell wall.

Although there are reports in the literature related to the extraction of chitin and its oligosaccharides from marine sources through chemical and enzymatic treatments, there are scarce studies with fungi. Based on the above, the objective of this work was to extract chitin fragments from *A. alternata* by alkaline and enzymatic treatments, as well as to analyze the physical–chemical characteristics of the whole chitin and its fragments. Our findings show that the enzymatic treatment was better than the alkaline treatment to extract chitin and its fragments of low molecular weight (<1 kDa) from *A. alternata* with high DA (76%). Therefore, the enzymatic treatments show promising results in the extraction process of chitin fragments from *A. alternata* without severely affect the DA of the molecule.

EXPERIMENTAL

Fungal Culture

The fungus *A. alternata* was obtained from the Plant Biotechnology and Postharvest laboratory of the CTAOV-CIAD, A.C. This strain was previously isolated from tomato fruits infected with the fungus, purified, identified by taxonomic keys reported by Lawrence *et al.*,[15] and those reported by Pryor and Michailides[16] for *A. alternata* strain (EGS 34-016, GenBank accession number AF347031.1), and maintained on potato dextrose agar (PDA) under refrigeration. The strain was inoculated on PDA (Difco Laboratories, Detroit, MI) and after 12 days of growth, it was used for the preparation of a conidial suspension of 10^7 conidia/mL. An aliquot of this conidia suspension (1 mL) was added to 100 mL of the potato dextrose broth (PDB) medium. The fungus was incubated at 27 °C, 150 rpm; light/dark (8 h/16 h) for nine days, according to method reported by Krishnaveni *et al.*[17] Each day the biomass was filtered through organza fabric, and dried for 24 h at 60 °C. The fungal biomass obtained was reported as g L^{-1} of dry weight (d.w.).

Extraction of Chitin with Alkaline Treatment

Chitin was extracted according to the procedure reported by Krishnaveni *et al.*[17] and Franco *et al.*[18] The extraction was carried out using sodium hydroxide (NaOH) at different concentrations (2 and 23.9%, w/v), with a ratio of 1:30 w/v, applying different temperatures (55, 75, 80, and 90 °C), and reaction times (1, 1.5, and 2 h). In the case of the sample applied with 2% (w/v) NaOH, 2 h, at 55 °C, a treatment with 0.91% (v/v) HCl was added (Treatment 5). The insoluble fraction was washed several times with distilled water and centrifuged at 15 000*g*. The pellet was recovered, lyophilized for 24 h, and stored until the physical–chemical analysis. A control sample was included which consisted of fungal biomass without any treatment.

Extraction of Chitin with Enzymatic Treatment

Enzymatic treatment was performed using a commercial protease from *Bacillus licheniformis* (Sigma-Aldrich Corp., St. Louis, MO) at concentrations of 2.4 and 24 U g^{-1} sample as reported by Sivan and Chet,[14] with slight modifications. Protease units were used as provided by the distributor. For this, 2 g of biomass was dissolved in 60 mL of 0.1 M phosphate buffer, pH 7.0 at 37 °C for 24 h. Subsequently, it was inactivated at 100 °C for 10 min, washed and centrifuged at 15 000*g* at 4 °C. The pellet was collected and dried for 24 h at 60 °C. The protein content was determined and reported as percentage of protein.

β-1,3-Glucanase from *Trichoderma longibrachiatum* (Sigma-Aldrich Corp.) at a concentration of 0.5 U mL^{-1} was used as reported by Jadahv and Gupta.[19] An enzymatic unit of β-1,3-glucanase was defined as the amount of enzyme that catalyzes the release of reducing sugar groups that are equivalent to 1 μmol of glucose/min. For this, 500 mg of biomass was dissolved in 50 mL of 0.5 M sodium citrate buffer, pH 3.5, at 27 °C. Enzymatic reaction times of 24, 48, and 72 h were used, and subsequently, the samples were washed and dried for 24 h at 60 °C. The influence of enzymatic reaction times on the characteristic bands of β-glucans was determined by infrared spectrophotometer with Fourier transform (FTIR).

In addition to the enzymatic treatments applied separately, the chitin was extracted using both enzymes to see their combined effect. For this, protease was applied, followed by β-1,3-glucanase according the methodologies previously described. The vibrational spectrum of the chitin extracted from *A. alternata* was determined by FTIR.

Production of Chitin Fragments from *A. alternata*

The biomass subjected to enzymatic treatments with protease and β-1,3-glucanase was suspended in H_2O (20 mL of H_2O for every 5 g of biomass) and subsequently passed to an ultrasonication process as reported by Takahashi *et al.*,[20] with slight modifications. A Branson ultrasonicator, model 2510, with a power of 250 W, for 3 h at 55 °C was used. Following this, an

ultrafiltration process was carried out using an Amicon stirred cell and Millipore regenerated cellulose membranes of NMWL cut-off size of 1 and 10 kDa (Sigma-Aldrich Corp.).

Initially, all the solution ultrasonicated was passed through the 10 kDa membrane disc to remove the high-molecular-weight fractions. As a final step, the permeate was passed through the 1 kDa membrane disc, collecting these low-molecular-weight fractions, named as F1. This fraction was characterized in base of the DA, protein, and glucosamine content. All the experiment was repeated twice.

Physical–Chemical Characterization of the Polysaccharides Extracted by Alkaline and Enzymatic Treatments

Analysis by FTIR Spectroscopy. An FTIR Thermo Scientific Nicolet, model iS50, coupled with a total attenuated reception (ATR) accessory was used. The samples were placed on top of the diamond crystal of the ATR–FTIR spectrophotometer. The mechanical pressure was applied from a rotary knob to the samples to acquire the proper contact with the diamond crystal. The vibrational spectra were recorded under environmental conditions. A sample sweep was performed at a standard resolution of 0.09 cm^{-1}. The spectral values were obtained as frequency, intensity, and band area, with a detection range of 400–4000 cm^{-1}, according to the method reported by Gnanasambandam and Proctor.[21]

Determination of the DA. The DA was determined by the following eq. (1) according to the methodology reported by Brugnerotto *et al.*[22]

$$DA = 31.92 \times \frac{A_{1320}}{A_{1420}} - 12.2 \quad (1)$$

where A_{1320}/A_{1420} are the ratio of absorbance recorded in the regions of 1320 and 1420 cm^{-1}, respectively.

Determination of Proteins Content by Micro Kjeldahl. Fungal sample (0.2 g) was taken and the estimation of proteins content was determined by Micro Kjeldahl according to the technique reported by Nielsen *et al.*[23] The total nitrogen content was affected by a factor (depending on the type of sample) to calculate nitrogen of a protein nature, being 6.25 according to that reported by Magomya *et al.*[24] The determinations were made in triplicate, and the results were reported as percentage of protein content.

Characterization of Chitin Fragments from *A. alternata*

Determination of the DA. The DA of the soluble chitin fractions was determined by conductometric titration as reported by Farris *et al.*[25] For this, the volume of the 0.1 N HCl titrant used to achieve the protonation of the free amino groups of the chitin molecule was determined. The determinations were made in triplicate, and the results were obtained according to eq. (2), and reported as percentage of DA.

$$DA\,(\%) = \frac{Molec_{HCl} \times MW\ GlcN \times 100}{Chitin\ MW} \quad (2)$$

where $Molec_{HCl}$ is the number of molecules of the HCl titrant used to achieve the protonation of the free amino groups of the chitin molecule and MW GlcN is the molecular weight of GLcN.

Glucosamine Content. The glucosamine content was determined by ultraviolet-visible spectrophotometry at 330 nm using a HACH spectrophotometer model DR500, according to the methodology described by Suárez *et al.*[26] Quantification was performed with a standard curve prepared with solutions of glucosamine at different concentrations, with an R^2 of 0.992. The determinations were done in triplicate, and the results were reported as µg of glucosamine/mg d.w.

Proteins Content. The protein content was estimated according to the method reported by Nielsen[23] previously described.

Statistical Analysis. A completely randomized design and a one-way analysis of variance were performed. For the means comparison, the Tukey multiple range test was applied with a confidence level of 95%, using the NCSS statistical analysis software (2010; NCSS, Kaysville, Utah).

RESULTS AND DISCUSSION

Figure 1 shows the results of the biomass production of *A. alternata* in PDB medium. As can be seen, after 9 days of growth, 7.03 g L^{-1} d.w. of biomass was obtained, and this time was selected for the subsequent growth of *A. alternata*. This value is similar to that reported by Koley and Mahapatra,[27] for *Alternaria solani* with biomass values of 9.0 g L^{-1} d.w. after 8 days of inoculation. On the other hand, Devi *et al.*[28] had reported biomass value of 20.2 g L^{-1} of *Alternaria helianthi* (infecting sunflower) in a growth time of 20 days. It is possible that these differences in biomass values could be attributed to the distinct species of *Alternaria* used in those studies.

Physical–Chemical Characteristics of the Fungal Chitin Extracted by Alkaline Treatment

The infrared absorption spectrum of chitin extracted by alkaline treatment (2% NaOH, 2 h, 90 °C) is shown in Figure 2. The polysaccharide presented its characteristic bands in the region of 3450 cm^{-1}, associated with OH bonds, as well as the CH bonds, were observed around the region of 2920 and 2850 cm^{-1}. The bands related to the amide and polysaccharide bonds were observed in the region of 1650–1050 cm^{-1}. The amides show two absorption bands, Amides I and II, at 1700–1500 cm^{-1} due to the vibratory coupling between the stretching frequencies of C=O and the deformation of NH. In the region between 1420 and

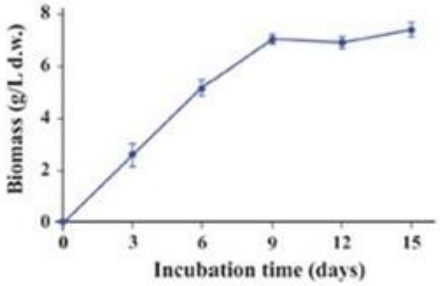

Figure 1. Production of biomass (g L^{-1} d.w.) of *A. alternata* in PDB medium. Each point represents the average of three replicates ± standard deviation. [Color figure can be viewed at wileyonlinelibrary.com]

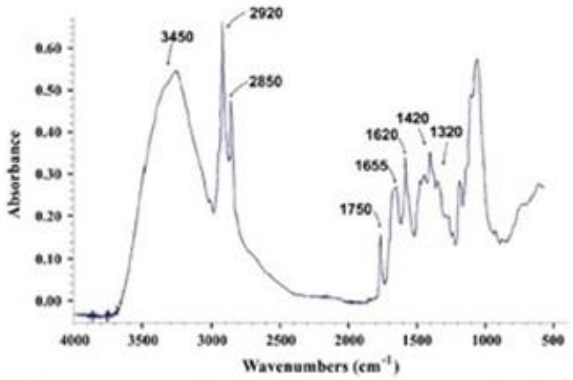

Figure 2. FTIR spectrum of *A. alternata* chitin extracted by alkaline treatment [2% (w/v) NaOH, 2 h, 90 °C], with characteristic chitin pattern between 1420 and 1320 cm^{-1}. [Color figure can be viewed at wileyonlinelibrary.com]

1320 cm^{-1} a similar pattern to chitin is observed. This pattern coincides to that reported by Gupta *et al.*[29] for *A. alternata*. Similar FTIR spectra of the samples extracted with 2% NaOH at 75 °C were obtained.

Different authors have reported the FTIR spectra of chitin and chitosan, which have very similar bands pattern. They reported that in the case of chitosan, the band of 1650 cm^{-1} is smaller, which is associated with the acetyl groups that the molecule possesses. However, to associate this behavior with the type of polysaccharide, it is necessary to determine the degree of acetylation.[30]

The polysaccharide extracted from the fungus by alkaline treatment (2% [w/v] NaOH, 2 h, 90 °C) presented 7% of DA. This low percentage of acetylation may suggest that the sample extracted with alkaline treatment is chitosan and not chitin. This result could be due to the aggressiveness of the applied chemical treatment.

Likewise, the polysaccharide extracted from *A. alternata* presented a glucosamine content of 77.86 μg mg^{-1} d.w. This result was similar to that reported by Fernandes *et al.*,[31] who obtained 100 μg mg^{-1} d.w. in *Alternaria infectoria*. The protein contents showed significant differences ($p \leq 0.05$) among the different conditions of the alkaline treatments carried out (Table I). The protein content found in the control sample was lower than that reported for filamentous fungi (20–30%); however, this may vary depending on the type of fungus and the culture medium used for its growth.[14] Despite obtaining a decrease in protein contents when the alkaline treatment was applied, the DA (7 and 8%) could be determined only when the sample was extracted with 2% (w/v) NaOH, 2 h, 90 °C, and 2% (w/v) NaOH, 2 h, 75 °C (Table I). In the other conditions of the alkaline treatments, the FTIR spectra showed bands in the region between 1320 and 1420 cm^{-1}, that could be attributed to co-extracted compounds (spectra not shown), which did not allow to get a defined patterns of fungal chitin.

These results suggest that the alkaline treatments were not effective to extract chitin from *A. alternata*, and do not coincide with those reported by other authors who extracted chitin from shrimp exoskeleton using strong alkaline conditions. This difference in our results could be associated with the differences in the composition between the shrimp exoskeleton and the fungal cell wall. In studies carried out with ground and deep-water shrimp, it was reported that the exoskeletons of both species possess the same microstructures: chitin, calcite, and other components, which are arranged in a helical structure in layers.[32] In both cases, it is stated that the shrimp exoskeleton is hard and resistant, containing mainly chitin, proteins, and minerals; especially Ca, P, Na, and Zn. It has amino acids such as glutamic acid and saturated and unsaturated fatty acids. It has been reported that its composition ranges from 15 to 20% chitin, 25 to 40% protein, and 40 to 55% calcium carbonate; therefore, initially require a demineralization process followed by deproteinization to obtain chitin.[33] Fungi, on the other hand, have different components in their cell wall. It is formed by an outer layer of proteins that oscillates between 20 and 30%, then a layer of β-glucans that oscillates between 50 and 60%, and in the innermost part is the chitin that represents between 10 and 20% in filamentous fungi.[34]

Some studies report the chitin extraction from fungi such as *Fusarium solani*, *Aspergillus terreus*, and *Biometria* sp., using alkaline treatment under 2% NaOH, 2 h, 90 °C, 1:30 w/v

Table I. Protein Content (%) and Acetylation Degree (%) of the Polysaccharides Extracted from *A. alternata* with Alkaline Treatment at Different Conditions

	Extraction conditions				
Treatment	Temperature (°C)	Time (h)	NaOH concentration (% w/v)	Protein content (%)**	Acetylation degree (%)
1	90	2	2	11.09 ± 0.79^{d}	7
2	80	1.5	23.9	13.85 ± 1.02^{c}	ND
3	75	2	2	12.75 ± 0.15^{c}	8
4	55	2	2	14.22 ± 0.76^{b}	ND
5	55	2 1/4	2 HCl 0.91*	15.40 ± 0.17^{a}	ND
Control	-	-	-	15.99 ± 0.10^{a}	ND

ND, undetermined.
*Treatment with HCl 0.91% (v/v) after treatment with 2% (w/v) NaOH, 2 h, 90 °C.
**Means followed by the same lower case letters in the column do not differ significantly by the Tukey test ($p \leq 0.05$).

conditions.[17] The FTIR spectra obtained by the authors show a behavior similar to that obtained in our study; however, in our study, a low degree of acetylation was obtained. This suggests that the alkaline conditions combined with high temperature for prolonged periods of time favors the release of acetyl groups from the chitin molecule, making it an inadequate treatment for extracting chitin from *A. alternata*, so other treatments must be used.

In the present study, enzymatic treatments were used as an alternative treatment to extract chitin from *A. alternata*, without severely affects the release of acetyl groups of the polysaccharide. The use of protease treatments at concentrations of 2.4 and 24 U g^{-1}, produced biomass with an estimated protein content of 10.38 ± 0.28 and 14.42 ± 0.28%, respectively (Figure 3). These values indicate a significant ($p \leq 0.05$) decrease in protein content of 10 and 29%, respectively, compared with the control.

These results are similar to those reported by Colina *et al.*,[35] who state that when applying enzymatic treatments to fungi, the protein content was reduced to a residual protein comprised between 1 and 7%. Cai *et al.*[36] found a decrease in protein content of 59.9% when applying protease at a concentration of 51 U g^{-1} in fresh mycelia of *A. niger*. It is important to note that the concentration of enzyme used was 2.5 times higher than that used in the present study.

The enzymatic treatments performed with β-1,3-glucanase at concentration of 0.5 U mL^{-1} for 24, 48, and 72 h showed similar FTIR spectra (Figure 4). In these spectra, the band corresponding to 1750 cm^{-1} is observed, which represents the vibration of the bonds corresponding to carbonyl groups (C=O). This band is present in samples with β-glucan content, according to other study reported.[37]

In these enzymatic treatments, a decrease of β-glucans is observed as the incubation time increases. The areas under the band corresponding to 1750 cm^{-1} were 5.05, 4.40, and 3.16 cm^2 for 24, 48, and 72 h, respectively. From these results, it is clear that after 72 h of enzymatic degradation, a decrease in the intensity of the band recorded in the region of 1750 cm^{-1} is observed, which could indicate that the content of β-glucans decreased by the effect of the enzyme β-1,3-glucanase. These results agree with

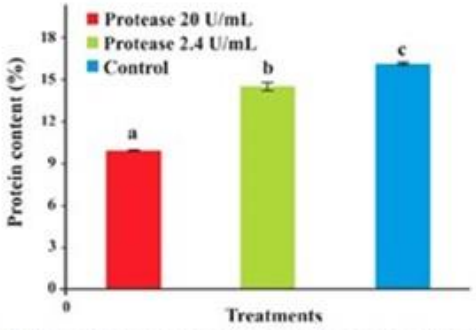

Figure 3. Effect of different concentrations of the commercial protease on the protein content in *A. alternata* biomass. Each column represents the average of three replicates and the bars represent the standard deviation. [Color figure can be viewed at wileyonlinelibrary.com]

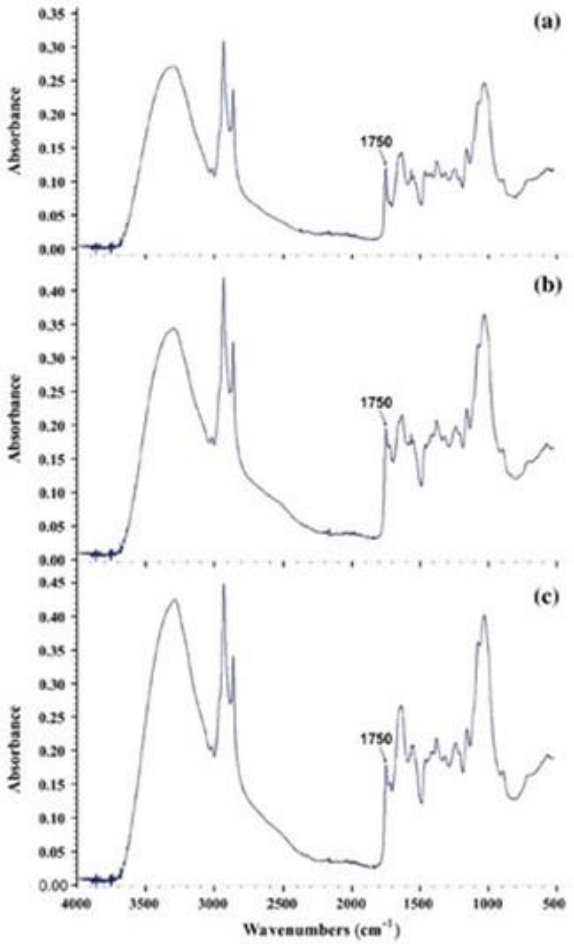

Figure 4. Spectrum FTIR of the *A.alternata* biomass degraded with β-1,3-glucanase enzyme at (a) 24, (b) 48, and (c) 72 h. [Color figure can be viewed at wileyonlinelibrary.com]

those reported by other authors who observed a decrease in the content of β-glucans when using enzymatic treatments with β-1,3-glucanase. In addition, it is important to mention that when applying high concentrations of the enzyme at high temperatures, greater reduction of β-glucans is obtained. Such is the case of De Marco and Felix[38] who reported β-1,3-glucanase from *T. harzianum* at a concentration of 1 U mL^{-1} and temperature of 60 °C, who get a higher reduction of β-glucans.

Figure 5 shows the FTIR spectrum of chitin obtained after the treatment with protease and β-1,3-glucanase. It can be observed that in the region corresponding to 1750 cm^{-1}, the bands corresponding to C=O bond are still present. Likewise in the region between 1280 and 1480 cm^{-1} unexpected bands are observed (band close to 1480 cm^{-1}), which could be attributed to co-extracted compounds to chitin, which did not allow to get a defined pattern of fungal chitin. Therefore, the DA could not be calculated.

However, the FTIR spectrum of chitin showed a similar behavior to other chitin spectra reported in the literature for different

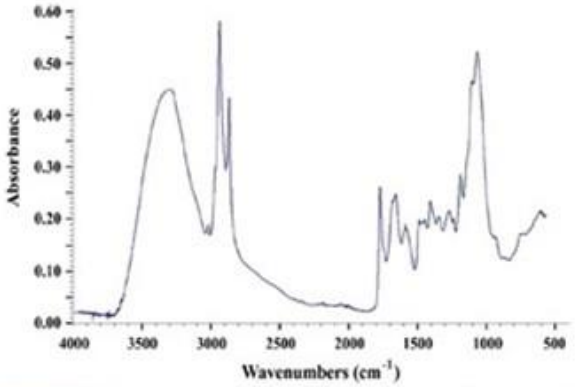

Figure 5. FTIR spectrum of *A. alternata* chitin extracted by enzymatic treatment combined with protease and β-1,3-glucanase enzymes. [Color figure can be viewed at wileyonlinelibrary.com]

fungi. Such is the case of those reported for *F. solani* and *A. terreus*,[16] and for chitin of *A. alternata* by Gupta *et al.*[29]

Physical–Chemical Characteristics of Fungal Chitin Fragments

After 3 h of the ultrasonication process, changes in the general appearance of the fungal sample and greater turbidity in the water were observed (Figure 6), which could be associated with the solubilization of the chitin fragments produced. Villa-Lerma *et al.*[39] reported a low depolymerization after sonication in a chitin sample of *Lecanicillium lecanii*, attributed to the vibratory movement of the molecular structure, which generates cavitation bubbles and production of energy.[40] Following the ultrasonication process, the sample was ultrafiltered, and the low-molecular-weight fraction (≤1 kDa) was collected. It is important to note that the fungal material obtained by alkaline treatment was insoluble in water (Figure 6), and was not possible to produce the chitin fragments with the ultrasonication process.

Figure 7 shows the percentage of DA of the fungal chitin fragments of low molecular weight (F1, ≤1 kDa), with 76 % of DA. The graph shows the pH and conductivity (χ) versus volume of HCl consumed. As HCl is added, NH_2 groups are protonated by binding to H^+ ions, while Cl^- anions will increase the conductivity.

Fungal chitin fragments (F1) showed an DA of 76%, which suggests that the enzymatic treatment was effective to extract chitin from *A. alternata* by not deacetylating the molecule as did the alkaline treatment. This result agrees with that reported by

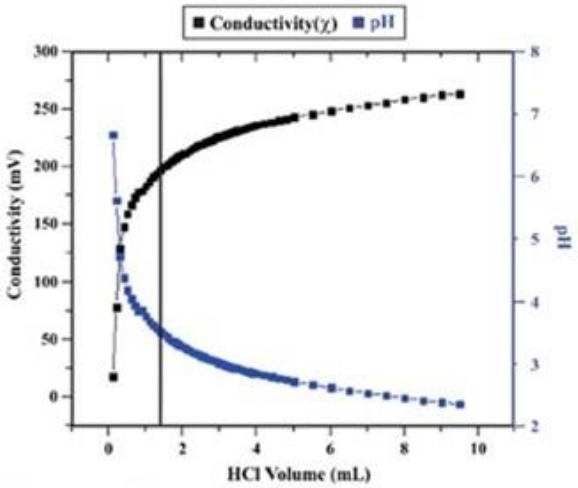

Figure 7. Conductometric titration of chitin fragments from *A. alternata* of low molecular weight (F1, ≤1 kDa), with 76% DA. The black line shows the point at which all free protonated amines are found, at which point of the changes in pH and χ between one measurement and another become smaller. [Color figure can be viewed at wileyonlinelibrary.com]

Kumar,[41] who points out that the process of recovering chitin by chemical method using acids and concentrated bases to deproteinize and demineralize the sample at high temperature can deteriorate the physicochemical properties of this biopolymer and, consequently, its biological properties. In that study, the author emphasizes the recovery of chitin from marine debris; this source is the most studied. Hence, it is necessary to continue studying the behavior of these extraction processes to get chitin from fungal sources.[41]

The glucosamine content found in F1 was 35.4 μg mg^{-1} d.w. This value was lower than that found in the fungal sample before ultrasonication and ultrafiltration processes (168.26 μg mg^{-1} d.w.). The glucosamine content of the biomass obtained before the ultrafiltration process was similar to that obtained in the alkaline treatment and that reported by Fernandes *et al.*[31] Considering the glucosamine content determined in F1 corresponding to low molecular weight fractions (≤1 kDa), it is suggested that this chitin fragment present a polymerization degree around 5, but further research is needed to confirm this data.

Regarding the protein content, no presence of these compounds was detected in the F1, suggesting that the enzymatic process

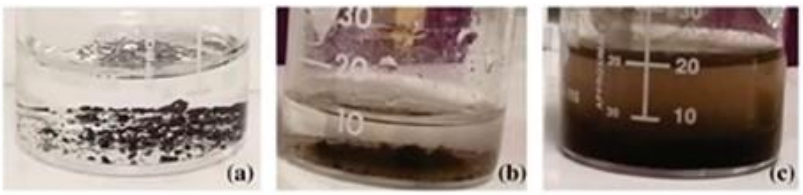

Figure 6. General appearance of chitin extracted by (a) alkaline, and enzymatic treatment, (b) before and (c) after ultrasonication process. [Color figure can be viewed at wileyonlinelibrary.com]

using proteases was effective to eliminate those proteins of low molecular weight.

CONCLUSIONS

The application of alkaline treatments to extract chitin from the fungus *A. alternata*, resulted in a polymer with low percentage of acetylation, with high protein content and possibly other compounds such as β-glucans.

The enzymatic treatments with protease at 24 U g^{-1} significantly decrease the content of proteins present in the sample. A decrease in the bands corresponding to β-glucans was observed when applying β-1,3-glucanase, as well as bands similar to those of fungal chitin. Fragments of fungal chitin with size ≤1 kDa (F1), 76% acetylation, and a glucosamine content of 33.6 μg mg^{-1} d.w. were obtained. The presence of proteins in F1 was not detected.

Therefore, it can be concluded that the enzymatic treatments show promising results in the extraction process of chitin fragments from *A. alternata* without affecting, the DA of the molecule.

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4. CHITIN FRAGMENTS FROM *Alternaria alternata* AS POSSIBLE ELICITORS OF PR3 ACTIVITYAND *Chi1* GENE EXPRESSION IN TOMATO FRUIT

Artículo en revisión por la revista Chilean Journal of Agricultural Research.

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ABSTRACT

Tomato is an economically important crop worldwide; however, large postharvest losses are caused by the fungus *Alternaria alternata*. Among the control strategies proposed, the induction of natural defense mechanisms can be activated as a response to the recognition of specific pathogen associated molecular patterns, such as chitin. The objective of this work was to evaluate the effect of chitin fragments from *A. alternata* on the activity of chitinase (PR3); and the relative expression of the gene coding for that enzyme. Chitin fragments less than 1 kDa (F1) with an acetylation degree of 76% and degree of polymerization < 5 were applied to tomato fruit, and samples were taken after 0, 0.25, 0.50, 0.75, 1, 3, 6, 24, and 48 h of exposure. The chitinase activity was measured by the fluorometric method, and quantitative expression of the *Chi1* gene was determined by real-time PCR. F1 induced a significant increase in the activity of chitinase in tomato after 0.5 h. Higher relative expression of the gene encoding chitinase was also observed after 0.5 h. This relative expression was more significant at 0.5 h than at the other times evaluated, suggesting an early fruit response to fungal chitin fragments. It is concluded that chitin fragments from *A. alternata* can induce the activation of the defense response in tomato fruit.

Key words: *Alternaria alternata*, chitinase (PR3), fungal chitin fragments, gene expression, tomato fruit.

INTRODUCTION

One of the most common problems for the fruit and vegetable industry is the postharvest deterioration of fruits and vegetables since it represents crucial economic losses. Tomato is an economically important crop worldwide, being Mexico one of its primary producers and the top exporter to the United States (FAO, 2020). However, the fruit is highly susceptible to spoilage caused by necrotrophic fungi such as *Alternaria alternata*, even under refrigerated storage conditions (Troncoso-Rojas and Tiznado-Hernández, 2014).

With the goal of controlling these postharvest pathogens, more effective and less environmentally harmful control strategies have been designed to ensure food safety (Troncoso-Rojas *et al.*, 2013). One of these strategies is the induction of plant defense mechanisms, including

the synthesis of secondary metabolites such as phytoalexins, the random creation of bonds between cell wall polymers, lignification, suberization, and the activity of enzymes related to pathogenesis such as chitinase and glucanase (Alkan and Fortes, 2015).

Among the molecules that induce natural defense mechanisms, chitin, a fungal cell wall component, and its oligosaccharides have been studied. In plants, it has been reported that chitin oligosaccharides that induce some defense responses have a degree of polymerization between 5 and 9 units of N-Acetyl glucosamine ($(GlcNac)_5$-$(GlcNac)_9$) (Iizasa *et al*., 2010; Liu *et al.*, 2012).

The induction of the defense response in plants begins with the recognition of pathogen-associated molecular patterns (Kawasaki *et al*., 2017). Subsequently, the transduction of signaling pathways mediated by molecules such as ethylene, jasmonic acid, and salicylic acid, induces the expression of defense genes in the nucleus, these genes encode proteins related to pathogenesis, such as chitinase (reported as PR3) (Ali *et al*., 2018). Roby *et al*., (1987) reported that melon plants exposed to chitin hexamers of shrimp exoskeleton induce chitinase activity at 6 h after the exposure, with maximum activity between 12 and 24 h post-treatment. The authors proposed that chitin oligosaccharides could be essential elements of communication in plant-pathogen interactions. Sun *et al*., (2018) on the other hand, found higher expression of the gene that codes for chitinase (*LeCHI9)* in tomato fruit treated with a solution of chitin obtained from yeast.

Although there are reports in the literature about the induction of defense mechanisms (through gene expression and chitinase activity) in plants by chitin oligosaccharides, this is the first report in which the effect of chitin isolated from fungi has been tested. Accordingly, the objective of this work was to examine the effect of chitin oligomers from *A. alternata* on chitinase activity and the expression level of the gene encoding chitinase in tomato fruit.

MATERIALS AND METHODS

Fungal chitin fragments

The chitin fragments were obtained from a strain of *Alternaria alternata* following the enzymatic method reported by Henry *et al.*, (2019). These chitin fragments showed a molecular weight of ≤ 1 kDa, with an estimated degree of polymerization of ≤ 5, an acetylation degree of 76.7 %, and an N-acetyl glucosamine content of 33.6 µg/mL.

Postharvest treatments of fungal chitin fragments in tomato fruit

Round tomato fruit in the pink stage of maturity was obtained from a central supply located in Sonora, México. According to the seller's information, the tomatoes were harvested the day before they were purchased. Fruits were selected if they were uniform in size, in color and free of visual damage or decay; fruits were disinfected with a 150 mg L^{-1} NaClO solution for 3 min. Then, they were rinsed with distilled water and divided into two batches. One of the batches was exposed to chitin fragments (F1) at a concentration of 0.05 μg mL^{-1} by immersion for 30 s, and the fruits were kept at 20 °C. The other fruit batch was immersed in sterile distilled water and was considered as the control. Samples from the tomato pericarp were taken after 0, 0.25, 0.5, 0.75, 1, 3, 6, 24, and 48 h of treatment. Samples were frozen at -80 °C and lyophilized in a freeze dryer (DC401/801, Yamato Scientific Co. Ltd., Tokyo, Japan).

Enzymatic activity of chitinase assay

A crude protein extract was obtained according to the protocol reported by Cota *et al*., (2007) with slight modifications. Briefly, a sample of lyophilized tissue was mixed with 100 mM sodium acetate buffer (pH 5.5) with 0.1% β-mercaptoethanol, and homogenized at 4 °C for 2 min in an homogenizer (Ultra TurraxT-25, IKA, NC, USA) with an 8 mm diameter stem. The sample was then filtered through organza fabric and centrifuged at 12000 x g at 4 °C for 20 min. The chitinase activity was quantified by a fluorometric method reported by Jiménez-Maldonado *et al*., (2018) with some modifications. The reaction mixture was prepared with the crude protein extract, 4-methylumbelliferone β-D-N, N', N-"triacylchitotrioside, and 50 mM sodium phosphate buffer, pH 7. The reaction mixture was incubated in a water bath at 37 °C for 5 min, and the reaction was stopped with 0.2 M Na_2CO_3. The release of 4-methylumbelliferone (4-Mu) was quantified with a standard curve using a Mini-Fluorometer (TBS-380, Turner Biosystems, CA, USA) in UV mode with a 325 nm excitation wavelength and 446 nm emission wavelength. Protein content was determined using the Bradford reagent according to the methodology described by Zhang *et al*., (2002) to express the chitinase activity as specific enzymatic activity. The units of chitinase enzymatic activity were defined as the release of 1 μmol of 4-Mu min^{-1}. The results were reported in U mg^{-1} of protein.

RNA isolation from the tomato fruits

Total RNA from tomato fruit was isolated following the methodology reported by López-Gómez and Gómez-Lim, (1992), which includes the use of LiCl to precipitate the RNA. The concentration and purity of the total RNA were determined by spectrophotometry using NanoDrop 1000 (Thermo Fisher Scientific Inc. DE, USA) equipment. The integrity of the total RNA was evaluated by 1% agarose gel electrophoresis using GelRed and Blue/Orange as charge buffer, and the presence of the 18S and 28S bands of the ribosomal RNA was used to ensure that the RNA was not degraded. The isolated RNA was dissolved in 30 μL of DEPC and stored at -80 °C.

Quantitative expression of the *Chi* gene using real-time PCR

A sample of total RNA was cleaned with a DNase RQ1 kit (Promega), following the manufacturer's instructions. After this step, first-strand cDNA was synthesized from clean total RNA using a SuperScript® III Reverse Transcriptase kit (Invitrogen), following the manufacturer's instructions with some modifications. A sample of 500 ng of total RNA was mixed with 1 μL of Oligo dT, 1 μL of Alignment Buffer and 1 μL of the reverse primer for the chitinase gene (*Chi*1), to increase the amplification efficiency, as reported by Feng *et al.* (2012), and the reverse transcriptase MMLV-RT (SuperScript^{TM} III) was added. Quantification of the expression level was determined using HotStart-IT SYBR Green Affymetrix® in StepOne Applied Biosystems (Thermo Fisher). The reactions contained SYBR Green Master mix, 20 ng of cDNA, 250 nM concentrations of forward and reverse primers, and ddH_2O. The genes Actin, TIP41 (like family protein), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were measured as reference genes, and a dynamic range assay including fivefold serial dilution was used. Primers used in the assay are shown in table 1. The PCR conditions were 5 min at 95 °C, 35 cycles of 2 min at 95 °C, 30 s at 60 °C, and a dissociation assay. The relative quantification was calculated with three technical repetitions per sample using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

The chitinase activity data were analyzed by a completely randomized design with factorial arrangement, where factor A included the chitin fragments (F1) and the control, while factor B included the time after treatment. Two ways ANOVA was carried out and when it indicated statistical significance, the Tukey-Kramer multiple range test was applied with a confidence level of 95%. Expression level data were analyzed by a completely randomized design. One-way analysis of variance was performed, and the Tukey-Kramer multiple range test with a confidence

level of 95% was performed using the NCSS statistical analysis software (2010; NCSS, Kaysville, Utah).

RESULTS AND DISCUSSION

Effect of fungal chitin fragments on chitinase activity in tomato

Figure 1 shows the results of the chitinase activity in tomato fruit as a response to the low molecular weight chitin fragments ($\leq$ 1 kDa). An increase in the level of chitinase activity in tomato fruit exposed to fungal chitin fragments with respect to the control was observed. After 0.25 h, the level of chitinase activity was low in F1 and control, but a sudden increase in the activity was observed in tomato fruit exposed to chitin fragments at 0.5 h after exposure ($p<0.05$) compared to the control and the other time points of the bioassay. A 10.4-fold higher chitinase activity than the control was induced at 0.5 h in the F1 treatment, demonstrating a quick defense response in the fruit. In agreement, Shen *et al*., (2017) reported that the activation of biochemical events occurs within 5 min after contact between the fungal pathogen and the plant. However, the early response observed here does not agree with other studies in which the induction of chitinase activity was reported within 12-24 h after exposure to yeast chitin (Sun *et al.*, 2018). Perhaps the different responses are due to molecular differences between the chitin of yeast and the chitin of fungi or to the differences in the molecular weight of these chitins.

An increase in chitinase activity stimulated by chitin treatments has been reported. Roby *et al*., (1987) found that melon plants exposed to chitin hexamers of shrimp exoskeletons showed an induction of chitinase activity at 6 h after exposure, with maximum activity between 12 and 24 h. In rice suspension cultures exposed to chitin oligosaccharides, a substantial increase in chitinase activity was observed after 3 days of culture (Inui *et al.*, 1997). Sun *et al*., (2018) observed an increase in enzyme activity after 12 h in tomato fruits exposed to chitin from *Saccharomyces cerevisiae* and inoculated with *Botrytis cinerea*, reaching maximum values after 24 h; however, no significant differences were reported between 12 and 24 h. From the abovementioned results, it is clear that chitin and its oligosaccharides act as elicitors stimulating the activity of one of the main pathogenesis-related proteins involved in the defense mechanisms in plants and in fruits. This may be an indication that other defense mechanisms are also induced by the chitin fragment tested in this study, although more experimental is needed to support this statement.

Quantitative expression of Chi1

The expression level of the gene coding for chitinase was quantified by real- time PCR after 0, 0.25, 0.50, 0.75, and 1h of exposure. These exposure times were selected based on the chitinase enzymatic activity results, and the data were normalized using GAPDH as a housekeeping gene, as this gene presented the best amplification efficiency (99.1%). cDNA fragments were successfully amplified using total RNA from tomato fruit. A single PCR band was generated and observed in agarose gel (figure 2). The band corresponding to the *Chi1* gene showed a size of 90 bp, which was the expected size according to the *in silico* analyses. A fragment of 150 bp was observed for the housekeeping gene (*GAPDH*). In the case of the negative control, these genes did not amplify, suggesting that the reaction was successful.

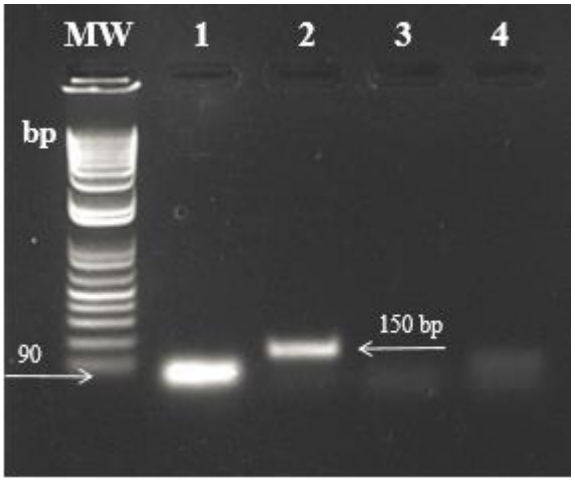

Figure 3 shows the relative expression of the *Chi1* gene in tomato exposed to the F1 treatment at different post exposure times. The expression level was upregulated when the fruit was exposed to fungal chitin fragments, showing significant differences in the expression level at the different post exposure times evaluated. After 0.5 h post exposure time, the maximum overexpression was observed (11.70-fold) with respect to the control, this result coincided with the higher level of chitinase activity obtained at the same exposure time. This expression level decreased at 0.75 h postexposure and was 1.96-fold higher than that of the control, being similar to that of 15 minutes and 1 hour. In agreement, Sun *et al*., (2018) reported that the gene *LeCHI9*, which encodes a chitinase enzyme, showed a higher expression level in tomato fruit treated with yeast cell wall chitin at all experimental times. Additionally, Brotman *et al*., (2012) reported that transgenic

Arabidopsis plants overexpressed the endochitinase chit36 when crab shell chitin or pentamer chitin oligosaccharide was applied.

It has been reported that chitin and its oligosaccharides induce the defense mechanisms when these molecules are recognized by plant membrane receptors and trigger a complex network of biochemical and molecular events to protect the plant against abiotic or biotic stress (Kawasaki *et al*., 2018, Liao *et al*., 2018). Some studies have shown that the degree of polymerization (dp) of chitin oligosaccharides is decisive in the recognition by plant membrane receptors and therefore in the activation of defense mechanisms (Inui *et al*., 1997; Zhang *et al*., 2002; Iizasa *et al*., 2010). According to these studies, the degree of polymerization for eliciting the plant immune system has been shown to be between 5 and 8 units of N-acetyl glucosamine, because these compounds are more chemically stable. The results observed in the present study agree with those of previous studies. In rice suspension culture, the elicitor activity of chitin oligosaccharides with different dp (1-6) was evaluated. The authors found that N-acetylchitopentaose and N-acetylchitohexaose showed a potent elicitor activity, causing a substantial increase in chitinase activity (Inui *et al*., 1997). Zhang *et al*., (2002) showed that chitin oligomers with dp between 6–8 were the most effective at inducing the expression of three chitin-responsive genes, while chitin oligomers with dp of 4-5 showed significantly lower induction. Iizasa *et al*., (2010) observed that chitin oligosaccharides with a dp of 5 to 9 (r $(GlcNac)_5$- r $(GlcNac)_9$) were recognized with great efficiency by the CERK1 receptor of *Arabidopsis thaliana*. Oligosaccharides with a lower degree of polymerization do not show affinity for the CERK1 receptor in *A. thaliana.*

CONCLUSIONS

These results suggest that chitin fragments from *A. alternata* with a molecular weight of ≤ 1 kDa, a polymerization degree of ≤ 5, and acetylation degree of 76% act as elicitors, thereby increasing the chitinase activity and the expression level of *Chi*1, which could be a response mechanism against fungal pathogens such as *A. alternata* in tomato fruits.

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Tables and Figures

Table 1. Primers used for the real-time PCR.

Gene symbol	Locus description	Oligo Sequence Forward/Reverse	Fragment size (*in silico* PCR)
Chi1	Chitinase 1	5’- CATGAAACTACGGGTGGATGGG- 3’ 3’- TCTCCAGGACTTCCTTGTTCCTG- 5’	90 bp
Actin	Actin	5’- TGGGATGATATGGAGAAGATATGG-3’ 3’- GGCTTCAGTTAGGAGGACAGGA- 5’	90 bp

GAPDH	Gliceraldehyde -3-phosphate dehydrogenase	5'-GTGGCTGTTAACGATCCCTT-3' 3'-AAGCTACTCTTCGGTCAGTG- 5'	150 bp
TIP41	Like family protein	5'- GCTGCGTTTCTGGCTTAGG- 3' 3'- ATGGAGTTTTTGAGTCTTCTGC- 5'	235 bp

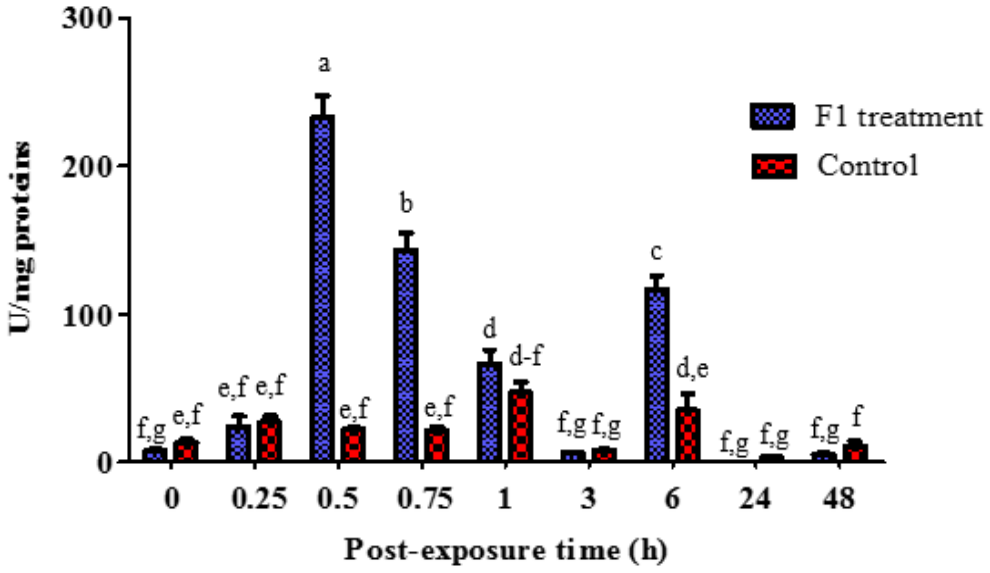

Fig. 1. Chitinase activity in tomato fruit exposed to *A. alternata* chitin fragments (F1) at different postexposure times (0, 0.25, 0.5, 0.75, 1, 3, 6, 24 and 48 h). Values presented are means ± SE. Different letters denote statistically significant differences (Tukey-Kramer test; at $P < 0.05$, $n = 6$).

Fig. 2. Electropherogram of the PCR results of cDNA amplification from tomato samples exposed to chitin fragments for 0.5 h, using specific primers. Lane MW: molecular weight marker (Kb plus DNA ladder); Lane 1: amplification of the chitinase (*Chi1*) gene; Lane 2: amplification of the

housekeeping gene glyceraldehyde 3-phosphate dehydrogenase; Lane 3: amplification of the negative control of *Chi* gen; Lane 4: amplification of the negative control of *GAPDH* gen.

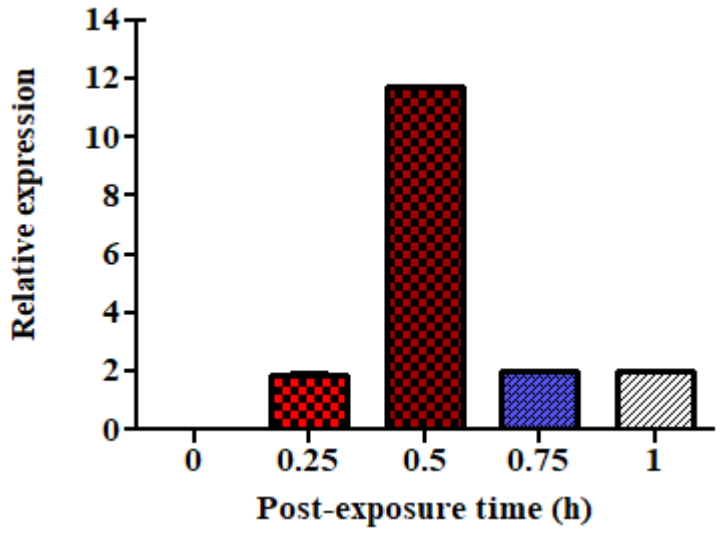

Fig. 3. Changes in the expression of the chitinase gene in tomato fruit exposed to chitin fragments from *A. alternata*, at 0, 0.25, 0.5, 0.75, and 1 h after exposure. Values presented are means ± SE. Different letters denote statistically significant differences (Tukey-Kramer test; at $p \leq 0.05$, $n = 6$).

5. DIFFERENTIAL EXPRESSION ANALYSIS OF GENES ENCODING CEBIP/CERK1 RECEPTORS IN TOMATO FRUIT USING RNA SEQ

Artículo en preparación

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ABSTRACT

Tomato is an economically important crop worldwide. The production of this fruit is reduced due to a pathogenic fungus such as *Alternaria alternata*. As alternative to the chemical control, there is the induction of the natural defense mechanism in plants by molecules such as chitin. Chitin is a polysaccharide of the fungal cell wall, and it has been reported it is recognized by membrane receptors, which are involved in the activation of defense genes in plants. Due to the scarce information on the genes involved in fungal chitin perception in tomato fruits, the main objective of this study was to identify putative pattern recognition receptors-associated genes in tomato fruit that perceive chitin fragments from the necrotrophic fungus *A. alternata*, by using RNA-seq. Four genes were identified in tomato fruits that code for chitin receptors like protein kinase, which shows a high percentage of identity with respect to chitin elicitor receptor kinase 1 (*AtCERK1*) and Lysin motif receptor-like kinase (*LYK4)* reported in *Arabidopsis*, and with respect to LysM receptor-like kinase (*Bti9*) and Lysin motif receptor-like kinase (*SlLYK1*), reported in tomato plant. An overexpression of a tomato gene encoding a receptor which recognizes chitin (*Solyc01g098420.3*) was observed at 30 min after the treatment. Likewise, the perception of fungal chitin fragments induced the overexpression of genes involved in the signaling mediated by Jasmonic Acid (JA) and Ethylene (ET), activating the defense mechanism, which was reflected with the overexpression of genes that code for proteins related to pathogenesis.

Keywords: fungal chitin fragments, RNA-Seq, differential gene expression, tomato fruit

Introduction

Post-harvest diseases of fruits and vegetables represent one of the main factors that cause significant losses in the food industry. Worldwide, these losses are estimated in a range between 5 to 25% in developed countries and 20 to 50% in not developed countries (Kitinoja and Kader, 2015). Tomato is highly perishable and susceptible to pathogens attack, such as *Alternaria alternata*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Rhizopus stolonifer*, among others (Pane *et al*., 2016; Takao *et al*., 2016; Troncoso-Rojas and Tiznado-Hernández, 2014). For the control of these pathogens, various alternatives to the use of synthetic fungicides have been proposed (Troncoso-Rojas *et al*., 2013; Usall *et al*., 2016; Wan *et al*., 2020). However, the

activation of the natural defense mechanism stands out as an environmentally friendly and promising alternative.

When pathogens interact with the plant surface, the fungal molecules, known as pathogen-associated molecular pattern (PAMPs), are perceived by plant membrane receptors pattern recognition (PRRs). These PRRs in coordination with other associated proteins activate the signal transduction to the nucleus, leading to PAMP-induced immunity (PTI) (Antolin-Llovera *et al*., 2012; Trouvelot *et al*., 2014; Shinya *et al*., 2012; Malik *et al*., 2020). Chitin is an essential component of the fungal cell wall, considered as PAMPs, that is perceived by PRRs (Eckardt, 2008; Sánchez-Vallet *et al*., 2015), and activate the innate defense mechanism of plants (Pusztahelyi, 2018). Chitin and chitin oligosaccharides perception had been studied mainly in *Arabidopsis thaliana* and in *Oryza sativa* (rice). It had been demonstrated that *A. thaliana* perceived chitin oligosaccharides with a degree of polymerization of 6 to 8 units, by chitin elicitor receptor kinase 1 (CERK1) (Zhang *et al*., 2002). This receptor is one of the main chitin receptors found in *A. thaliana*. It is a member of the receptor like kinases (RLK) family. Further it is composed of three extracellular lysine domains involved in chitin perception, a transmembrane domain and a intracellular cytoplasmic kinase domain which can initiate a signaling cascade (Bi *et al*., 2018; Cao *et al*., 2014; Malik, 2020; Miya *et al*., 2007). Other proteins such as LysM receptor kinase and AtLYK5 in *Arabidopsis*, binds to CERK1 forming a heterotetramer complex which can activate the citoplasmic kinase domain to initiate the downstream defense responses (Cao et al., 2014; Desaki, *et al.,* 2018). By other side, the chitin elicitor-binding protein (CEBiP) receptor was identified in *O. sativa.* It consists of an extracellular lysine domain and one transmembrane domain. Further, it does not possess intracellular domains to start the signal transduction and that is why this receptor interacts with a RLK receptor (Kaku *et al*., 2006; Hayafune *et al.*, 2014; Buendia *et al*., 2018; Shinya *et al*., (2014).

Although these receptors have been extensively studied in some plants, the published information on chitin receptors with lysine domains that activate the immune response in tomato is very limited or absent, to our knowledge. In fact, the scientific information that exists refers to the chitin receptors that perceive synthetic chitin, or chitin form crustaceous, yeast, or bacteria sources (Castañeda *et al*., 2011). Besides, there are reports studying the genes encoding for receptors that are expressed in response to pathogens infections, or in response to arbuscular mycorrhiza, among which the genes that code for chitin receptors has been found. For instance,

Zeng *et al*., (2012) isolated the *Bti9* gene in tomato plant in response to AvrPtoB protein from *Pseudomonas syringae*. Based on the tomato genome sequence, the authors found three homologs of *Bti9*, designated as *SlLyk11*, *SlLyk12*, and *SlLyk13* that shares the same clade with *Bti9*, suggesting that these are involved in the molecular responses to PAMPs.

Similar results were reported by Liao *et al*., (2018), who identified in tomato plant four orthologous genes to CERK1 (*SlLYK1*, *SlLYK11*, *SlLYK12* and *SlLYK13)* that encode the receptor protein kinase CERK1. The authors found that *SlLYK1* gene expression was upregulated by chitin oligosaccharide; whereas *SlLYK12* was involved in *Arbuscular mycorrhizal* (AM) symbiosis, suggesting that those chitin receptor genes have distinct function in plant depending on the specific plant tissue, or PAMPs molecule. Taking into account the lack of information about PRRs in tomato fruit that perceive fungal chitin oligosaccharides, the main objective of this study was identify putative PRRs-associated genes in tomato fruit that perceive chitin oligomers from the necrotrophic fungus *Alternaria alternata*, through transcriptome analysis using RNA-seq.

Materials and Methods

Fruit Material

Tomato fruit of the Druck variety was obtained from a local market located in Hermosillo, Sonora, México. According to the seller's information, the tomatoes were harvested the day before they were purchased. Fruits were selected based on the uniformity in size, pink maturity stage (color number 4 according to color card of USDA), and free of visual damage or decays.

Fungal chitin fragments

Chitin fragments of low molecular weight (≤ 1 kDa) were obtained from *Alternaria alternata* as described elsewhere (Henry *et al*., 2019). These chitin fragments showed an estimated polymerization degree of ≤ 5, an acetylation degree of 76.7 %, and N-acetyl glucosamine content of 33.6 μg/mL

Application of the chitin fragments

Fruits were disinfected with a NaClO solution (150 μL/L) for 3 min. They were rinsed with distilled water to remove traces of chlorine and divided into two group. One of the fruit group was exposed to a solution of chitin fragments (F1) at a concentration of 0.05 μg/mL by immersion for 30 seconds. Thereafter, the fruits were kept at 20 °C during 30 minutes. The other fruit group was

immersed in sterile distilled water and was considered as a control. Based in a preliminary study conducted in our lab, it was observed a large enzymatic activity 30 minutes after the fruit was challenged with fungal chitin fragments. After 30 min of tomato exposition, samples of tomato pericarp were taken and frozen at -80 °C.

RNA isolation from the tomato fruits

Tomato tissue was frozen in liquid nitrogen and grind. Three biological replicates for each treatment were used. The RNA extraction was conducted according to the technique of López-Gómez and Gómez-Lim, (1992), which is based on the precipitation of RNA with lithium chloride. The RNA concentration was determined using a Nano-Drop ND-1000 UV-vis spectrophotometer (Nano-Drop Technologies Inc., Wilmington, DE, USA). The RNA purity was determined using Nano-Drop spectrophotometry based on the relationship of 260 and 280 nm. RNA integrity was analyzed by electrophoresis with 1% agarose to observe the bands at 18S and 28S of ribosomal RNA. The RNA was dissolved in 30 µL of water nuclease free and stored at −80°C.

RNA-Seq library construction and sequencing

RNA was used to prepare six independent libraries using TruSeq RNA sample preparation kit v2 LT following the manufacturer instructions (Illumina Systems). RNA concentrations were 197 – 214 ng/µL with an RNA integrity number of approximately 8. These libraries were run on an Illumina NextSeq, at the Laboratory of Genomic Services, CINVESTAV, Irapuato, Guanajuato, México (http://langebio.cinvestav.mx/labsergen/). Sequencing was done in single-end mode with 150 bp read length. I was generated approximately 20 million single reads per sample.

Quality analysis of reads and rRNA filtering of reads

The quality of the raw reads obtained from the sequencing of the transcriptome was carried out using FastQC program (Andrews, 2010). Only sequences with a quality higher than 30 in at least 85% of the entire read were selected. Subsequently, using the FASTX-Toolkit, all the sequencing adapters, rRNA reads, and low quality/shorter reads (Q value < 30/35 bp) were removed from the RNA-Seq data to ensure optimal mapping to the genome reference (New tomato reference genome SL4.0 and ITAG4.0, Solgenomics).

Mapping of the Short Reads to the Tomato Genome.

Short reads were mapped to the reference genome of the *Solanum lycopersicum* (Causse *et al*., 2016) (SolGenomics platform current version SL4.0 and ITAG4.0 annotation;

https://solgenomics.net/), using the software Bowtie2 (Langmead and Salzberg, 2012). The parameters used were a phred score of 30, average phred score of no less than 20 for the entire length of the reading, and a minimum size of 35 nucleotides. The rRNA database for *Solanum lycopersicum* was prepared based on the information available from the arb-silva database, and consisted of 270 sequences reported to date

Determination of Gene Differential Expression

To determine the levels of differential gene expression *in silico* between the control and the fruits exposed to chitin fragments for 30 min, massive RNA sequencing analysis was conducted. A comparative analysis of sample normalization methods was implemented in order to reduce the "bias" caused by the size of the libraries, sequencing depth and possible sequencer errors. This comparison allowed a more reliable expression profiles to be obtained during the analysis and exploration of results. The sample normalization methods applied were: DESeq (RLE: relative log estimate), TC (Total Counts, available in the edgeR package), RPKM (Reads per Kilobase Million), TMM (Trimmed mean M values), UQ (Upper Quartile) (Li *et al*., 2020). Of these methods, the TMM presented a more uniform distribution pattern and that is why this method was used to generate the Principal Components Analysis (PCA) analysis of the normalized samples to determine the grouping of the control and treatment samples.

The gene differential expression was calculated using the Cuffdiff program, belonging to Cufflinks v2.0.0, in RPKMs (Reads per kilobase of the exon per million fragments mapped) (Trapnell *et al*. 2012). Reads per kilobase per million and the *P*-value for differential expression using the log ratio statistic were calculated. A complementary analysis with 3 statistical methods contained in the edgeR and DESeq2 package were applied, which were Exact test, Generalized linear model (GLM) and *voom-limma* using the R Statistical Analysis package (Anders *et al*. 2012). The best results were obtained with the GLM and Exact test methods, registering a greater number of differentially genes expressed. The analysis was conducted to test the significance with a cutoff of $P < 0.05$ of probability.

Gene Expression by Real-Time Quantitative Polymerase-Chain-Reaction Analysis

Six differentially expressed genes (DEGs), characterized by interesting expression profiles in response to chitin fragments exposure, were selected for qRT-PCR (Table 1). For this, 1.0 µg of total RNA was cleaned with the DNase RQ1 kit following the manufacturer's instructions

(Promega). After this step, the first-strand of cDNA was synthesized from clean total RNA using the SuperScript® III Reverse Transcriptase kit according to the manufacturer's instructions (Invitrogen), with some modifications. Sample of 500 ng of total RNA was mixed with 1 μL of dNTP Mix, 1 μL of the reverse primers, in order to increase the amplification efficiency as reported by Feng *et al.* (2012) and the reverse transcriptase MMLV-RT (SuperScript^{TM} III) and sterile water. Quantification of the expression level was determined using HotStart-IT SYBR Green Affimetrix® in StepOne Applied Biosystems Thermo Fisher. The reactions contained 10 μL of SYBR Green Master mix, 60 ng cDNA, 1 μL of 10 μM concentration of forward and reverse primers, and ddH_2O. The gene glyceraldehyde-3-Phosphate-dehydrogenase (GAPDH) were examined as a housekeeping gene, and a dynamic range assay including five-fold serial dilution were used (Nolan *et al*., 2006). Primers used in the assay are shown in table 1. The relative quantification was calculated with three technical repetitions per sample using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Table 1. Primers used in gene expression analysis by RT-qPCR

Gene	Size (bp)	Sequence
Pr2-Fw	24	AAGTATATAGCTGTTGGTAATGAA
Pr2-Rv	21	ATTCTCATCAAACATGGCGAA
Pr5-Fw	20	GCAACAACTGTCCATACACC
Pr5-Rv	19	AGACTCCACCACAATCACC
SlBti9-Fw	24	AGACCACCTCCATCAGTATGGTCA
SlBti9-Rv	24	TGCCTGAAAGCACTGGAGAATTGC
GAPDH-Fw	20	GTGGCTGTTAACGATCCCTT
GAPDH-Rv	20	GTGACTGGCTTCTCATCGAA
Chi1-Fw	23	TCATGAAACTACGGGTGGATGGG
Chi1- Rv	23	TCTCCAGGACTTCCTTGTTCCTG
LYK4-Fw	20	GGGATCTGTTTATCGGGGCA
LYK4-Rv	20	TATCCCAGCTTTAGCGCCAC

Results and Discussion

The total number of clean single reads were of 81’343,998 for treatment and 93’935,830 for control. These clean reads were obtained after removing bases with low quality, rRNA, and adaptor sequences. More than 97% of clean reads were mapped (Tabla 2) using the reference genome of *Solanum lycopersicum* available on the Solanaceae Genomics Network website (https://solgenomics.net/). This web site consists of 13 genomic sequences with a total size of 782.52 Mb and an annotation of 34,075 genes, which were used for differential expression analysis.

Tabla 2. Transcriptome assembly samples.

Samples	**Biosample accesion number**	**Number or reads after trimming**	**% Mapped reads**
Fruit exposed to chitin fragments	RT1SL1SS01	25’098,062	97 %
	RT1SL1SS02	33’739,540	97 %
	RT1SL1SS03	22’506,396	98 %
Control fruit	RT1SL1SS04	31’459,284	97 %
	RT1SL1SS05	26’387,161	98 %
	RT1SL1SS06	36’089,385	97 %

Differential expression analysis

The transcriptomic analysis revealed 22,621 transcripts that were processed by Cuffdiff, and using the variable of FPKM, it was possible to find genes that were differentially expressed between treatment and control, always considering an adjusted *P*-value less than 0.05 and a *fold-change* greater than 0.5 as threshold. Figure 1 shows the Venn diagram, in which the total of differentially expressed genes (DEGs) were obtained by different methods. With the Exact test method, 240 DEGs were obtained (127 down-regulated and 113 up-regulated); while 286 DEGs were obtained (140 down-regulated and 146 up-regulated) with the GLM method, and with the Voom method 13 DEGs (1 down-regulated and 12 up-regulated) were obtained.

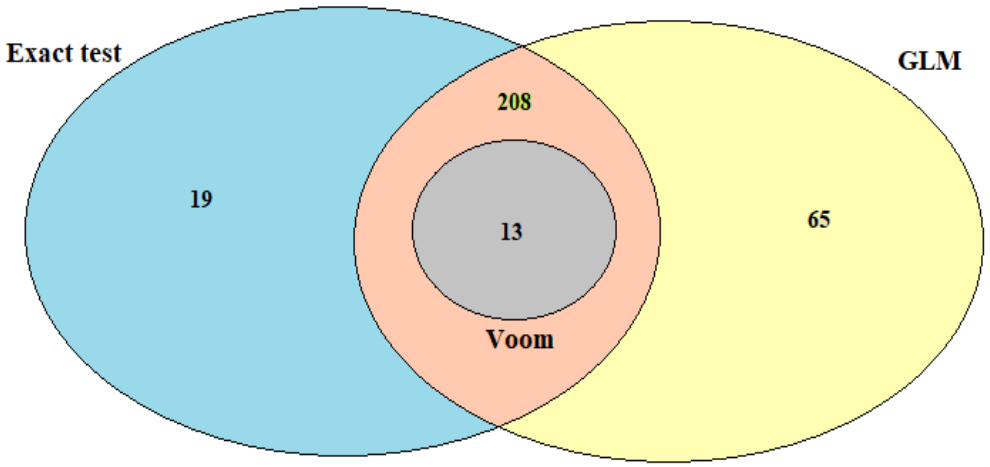

Fig 1. Differential genes expressed in tomato fruit in response to chitin fragments from *A. alternaria*, after 30 min post-treatment (Venn diagram).

The three methods together reported up to 305 DEGs. Up-regulated transcripts in response to fungal chitin fragments have roles in the metabolic process, biosynthetic process, and physiological process, such as the growth, maturation, respiration, among others. For instance, the over-expression of the constitutive genes ubiquitin (*Solyc00g011560.1*) and actin (*Solyc04g011500*) were observed, which have important functions within the cell, such as marking of other proteins to be degraded by the proteasome, participation in mobility and cell contraction during cell division, respectively (Sundvall, 2020). Additionally, other important genes, such as cytochrome P450, glutaredoxin family protein, NAC protein aspartic proteinase, calmodulin-like protein, subtilisin-like protease, Avr9/Cf-9, ethylene-responsive transcription factor, and acetolactate synthase (GO:0009082), which is specifically involved in the tomato resistance to powdery mildew (Gao *et al.*, 2014), were enriched in the GO terms.

Transcriptional response of genes encoding for chitin-binding receptors to Alternaria chitin fragments

It is important to highlight that some up-regulated transcripts in the treatment are involved with the chitin perception and defense responses. In the present study, *Solyc01g098420.3* gene was over-expressed 2.07 fold-time in response to fungal chitin treatment (Table 3). This gene putatively encode for a chitin receptor protein kinase, and present 99% of identity with the gene At5g59670,

which code for a receptor-like protein kinase in *Arabidopsis*. Based on the tomato genome (*Solanum lycopersicum*, http://solgenomicss.net), two more putative genes encoding for chitin receptor-like protein kinase were identified in tomato fruits in response to fungal chitin fragments. *Solyc07g049180.3* presented 81.05% of identity with *Bti9* and *SlLYK1* genes, which were previously reported in tomato plants (Zeng *et al*., 2002; Liao *et al*., 2018), and the gene *Solyc02g089900.1* with 84.4% of identity with *LYK4* gene previously reported in *Arabidopsis* (Wan *et al*., 2012). However, both genes were down-regulated at 30 min post-treatment. *Bti9* is a LysM-RLK gene that encodes a serine/threonine protein kinase domain with 76% amino acid identity to *Arabidopsis* CERK1 receptor. *Bti9* gene was identified in tomato plants to perceive AvrPtoB from *Pseudomonas syringae* (Zeng *et al*., 2012). In other study, Liao *et al*., (2018) found the over-expression of *SlLYK1* gene in tomato plants in response to chitin oligosaccharides with a polymerization degree of 8. In the same study, the authors found that *SlLYK1* gene as well as *SlLYK11*, *SlLYK12*, and *SlLYK13* genes, were in the same clade as the *CERK1* gene, concluding that those genes are orthologues with CERK1. Based on the NCBI data, *Bti9* and *SlLYK1* genes are highly similar and share the same function. So, based on these information and according to the studies reported in the literature, it could be inferred that *Solyc01g098420.3* gene identified in the present study is an ortholog to AtCERK1; while *Solyc07g049180* and *Solyc02g089900* genes are homologous to *Bti9* and *SlLYK1*.

As mentioned before, chitin oligosaccharides are perceived by the PRR? through chitin receptors protein kinase. This union occurs between the acetyl group of chitin and the lysine domains of the receptor, giving rise to the formation of homodimers and/or heterodimers (Ascencio *et al*., 2000; Kawasaki *et al*., 2017; Miya *et al*., 2007; Shinya *et al*., 2012 ; Buendia *et al*., 2018). Likewise, the participation of receptors like protein kinases with lysine domains (LYK) that interact with receptors type RLK had been reported. For instance, the AtLYK5 receptor in *Arabidopsis* was reported to bind to chitin with higher affinity than AtCERK1 (Wang *et al*., 2008; Cao *et al*., 2014). In that study, it was proposed that AtLYK5 functioned as the major chitin receptor, recruiting AtCERK1 to form a chitin-inducible receptor complex. Erwig *et al*., (2017) confirmed that CERK1 is essential for the recognition of chitin oligosaccharides in *Arabidopsis,* and that there are other receptor proteins important for this interaction to be effective. In the present study, genes encoding protein kinase receptors (RLK) and genes encoding protein kinase receptors with lysine domains (LYK) were identified. However, more studies are required to determine the receptor

characteristics, and which one has the highest affinity for fungal chitin fragments with a polymerization degree of ≤ 5.less or equal to 5.

Once chitin perception occurs, the signal is transmitted to the interior of the cell through the intracellular kinase domain, which interacts with proteins that activate the phosphorylation of MAPKs, triggering a complex network of biochemical and molecular events that induce the against biotic stress (Kawasaki *et al*., 2017; Yamada *et al*., 2016). According to AbuQamar *et al*., (2008) and Ray *et al*., (2015), the overexpression of the *TPK1b* gene was observed in tomato plants in response to the infection by *Botrytis cinerea* and *Alternaria solani*, respectively. It was observed that reducing the *TPK1b* gene expression, the plant's susceptibility to pathogens attack was increased. The authors concluded that this gene encodes for a homologous CERK1 receptor so it is possibly that is involved in the perception of fungal chitin.

Signaling molecules were upregulated by fungal chitin fragments

Another fundamental step that occurs within the plant pathogen interaction is the signals transduction mediated by signaling molecules such as ET, JA and salicylic acid (SA), of which the signaling by JA/ET activates the defense response against necrotrophic fungi (Li *et al*., 2019) such as *A. alternata*. Three calmodulin related DEGs were induced in response to fungal chitin fragments, suggesting the involvement of a Ca dependent signaling pathway. Furthermore, our findings show the key role of the hormones in tomato defense against biotic stress as observed by the genes involved in ET and JA pathways. Some DEGs related to ET responsive factors were found, including ethylene-responsive transcription factor-like proteins, and ethylene-responsive transcription factor ERF-like, among them an overexpression of the genes encoding the never ripe ethylene receptors (*NR, Solyc09g075440.4*), and ethylene receptor ETR4 (*Solyc06g053710.3*) was observed. In addition, some DEGs related to JA were found such as lipoxygenases (LOX, *Solyc03g122340, Solyc01g006540.4*), and allene oxide synthase (ACS, *Solyc04g079730.1*) involved in the biosynthesis of jasmonic acid, which were induced at 30 min after treatment (Table 3). These results agree with those reported by Alkan et al., (2015) who observed an overexpression in the NR gene with 2 to 24 times when inoculating green tomato fruits with *Collectotrichum gloesporioides*.

***Alternaria* chitin fragments perception induce changes in defense related proteins in tomato fruit**

According to the results obtained in the present study, the fungal chitin fragments activated the defense responses in tomato fruit. In the present study, the gene expression encode for proteins related to pathogenesis such as chitinase (*Chi1, Solyc02g061770*), endo β-1,3 glucanase (*PR2, Solyc01g008620.4*), thaumatine (*PR5, Solyc08g080670.1*), PR1 (*Solyc00g174330*), and polyphenoloxidase (*Solyc08g074680*), among others, were observed (Table 3). It is widely documented that the chitinases and β- 1,3 glucanases have been shown to have antifungal activity either *in vitro* and under *in vivo* conditions (Ali *et al*., 2018; Cota *et al*., 2007; Sanchez-Estrada et al., 2009). These results agree with that reported by Sun *et al*., (2018), who observed the overexpression of the genes that encode chitinase (*LeCHI9*), PR2 (*LePRb*), and phenylalanine ammonium-lyase (*LePAL*) in tomato fruits in response to chitin of *Saccharomyces cerevisiae*, compared to the control. *LePR2b* in chitin-treated fruits was 19.3 times higher than in the control at 24 h, while *LeCHI9* was 2.2 times at 12 h and 6.3 times at 48 h, respectively. The authors suggested that increased enzymatic activities and transcriptional levels of GLU and CHI may be an important mechanism induced by yeast cell wall chitin to prepare fruit for increased disease resistance. On the other hand, Alkan *et al*, (2015) found that the defense genes regulated by ethylene increased substantially in tomato fruit inoculated with *Collectotrichum gloesporioides*. The authors found the overexpression of the genes encoding chitinases class 1 (> 500 times; *Solyc07g009510.1*), the protein related to pathogenesis 1 (PR1, > 1000 times; *Sol-yc09g091000.2*), and PR 10, (> 100 times, *Solyc09g090990.2*).

Taken together, the results of the present study indicate that chitin fragments from *A. alternata* induced an overexpression of the genes encoding for chitin binding receptor like protein kinase, activating the defense response in tomato fruits.

Table 3. Detailed information of differential genes expressed related to chitin receptor in tomato fruit exposed to *Alternaria* chitin fragments at 30 min.

Gene	ID	GO name	GO	Pathway name	Class
SlCERK1	*Solyc01g098420.3*	Receptor-like protein kinase	GO:0019199	Transmembrane receptor protein kinase activity	Molecular function
Bti9	*Solyc07g049180.3*	Receptor-like protein kinase	GO:0019199	Transmembrane receptor protein kinase activity	Biological process
LYK4	*Solyc02g089900.1*	Receptor-like kinase, Serine/threonine protein kinase	GO:0006468	Protein phosphorylation	Biological process
			GO:0016998	Cell wall macromolecule catabolic process	Biological process
RLK	*Solyc02g080010.2.1*	Receptor like protein, putative resistance protein with an antifungal domain	GO:0006468	Protein phosphorylation	Biological process
LysM	*Solyc01g099130.3*	LysM domain containing protein	GO:0016998	Cell wall macromolecule catabolic process	Biological process
ERF2	*Solyc03g093560.1.1*	Ethylene-responsive transcription factor 2	GO:0003677	DNA binding	Molecular function
			GO:0006355	DNA binding	Molecular function
AOS	*Solyc04g079730.1*	Allene oxide synthase	GO:0009978	Allene oxide synthase activity	Molecular function
CALM1	*Solyc02g094000.1.1*	Calmodulin-like protein	GO:0005509	Calcium ion binding	Molecular function
NAC	*Solyc06g073050.2.1*	NAC domain protein	GO:0045449	Regulation of transcription	Biological process
SBTs	*Solyc02g092670.1.1*	Subtilisin-like protease	GO:0006508	Proteolysis	Biological process
			GO:0043086	Negative regulation of catalytic activity	Biological process
CYPs	*Solyc07g006890.1.1*	Cytochrome P450	GO:0020037	Oxidation-reduction process	Biological process
			GO:0055114	Oxidation-reduction process	Biological process
GLRX	*Solyc02g087850.1.1*	Glutaredoxin family protein	GO:0045454	Cell redox homeostasis	Biological process
	Solyc02g080540.1.1	ATP synthase gamma chain	GO:0045261	Proton-transporting ATP synthase complex	Celular component
GLUC	*Solyc01g008620.4*	B-1,3-glucanase	GO:0008810	Cellulase activity	Molecular function
Chi2	*Solyc07g009510.1*	Chitinase type II	GO:0008843	Endochitinase activity	Molecular function

Validation of the results obtained in silico by qRT-PCR

Figure 2 shows the relative expression level by qRT-PCR of the genes encode for chitin receptors and some pathogenesis-related proteins in tomato exposed to fungal chitin fragments during 30 min. It was found a large expression level in Bti9 (*Solyc01g098420.3*, 6.27-fold) when it was measured by qRT-PCR method; while *SlLYK1* and *LYK4* registered a similar expression level (2.57 and 2.85-fold, respectively).

Experimental gene expression results from qRT-PCR were similar with the RNA-seq differential expression data. Figure 3 shows the expression ratios between tomato fruit exposed to fungal chitin fragments and untreated tomato fruit (control) for some of the selected genes. Profile of gene expression by RNA-seq and qRT-PCR are similar and revealed a high correlation between methods for transcription analysis.

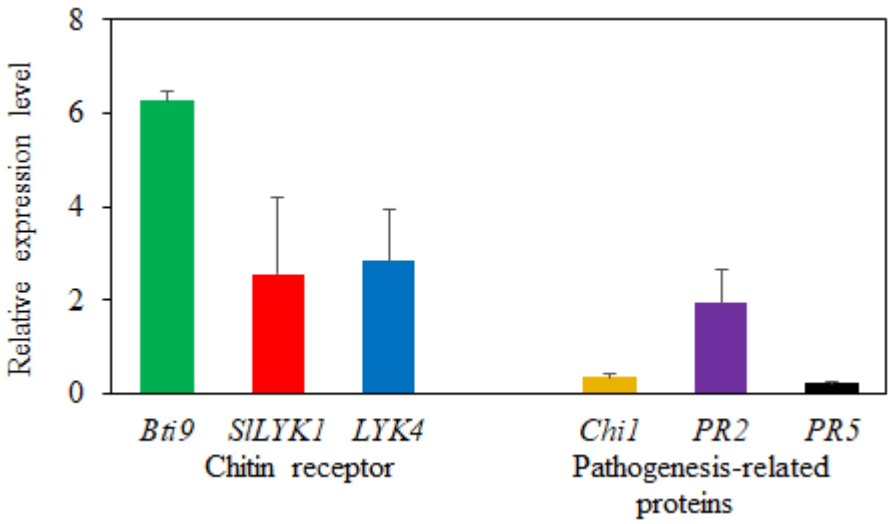

Figure 2. Relative expression level evaluated by qRT-PCR of the genes encode for chitin receptors and some pathogenesis-related proteins in tomato fruit exposed to *Alternaria* chitin fragments during 30 min.

Experimental gene expression results from qRT-PCR showed high level of correlation with the *in silico* RNA-seq differential expression data. Figure 3 shows the linear regression graph between tomato fruit exposed to fungal chitin fragments and untreated tomato fruit (control) for some of the selected genes. Profile of gene expression by RNA-Seq and qRT-PCR are similar and revealed a high correlation between methods for transcription analysis. Linear regression analysis showed a R^2 value of 0.79 indicating a close correlation between transcript abundance quantified by qRT-PCR and the transcription profile obtained by RNA-Seq data, which supports the precision and robustness of the data.

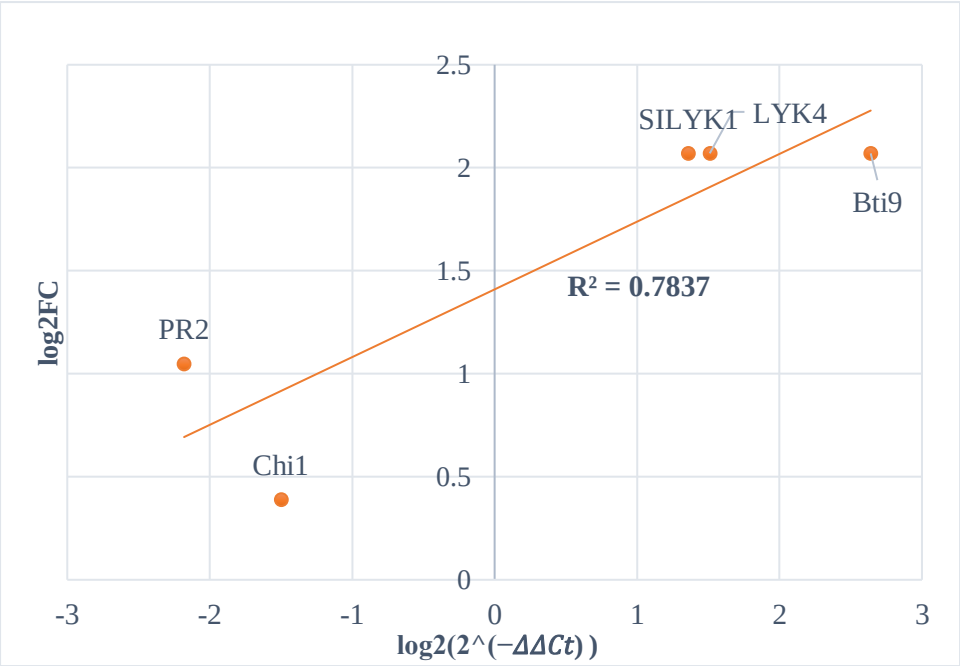

Figure 3. Lineal regression analysis between the gene expression of genes induced in tomato by the chitin treatment calculated *in silico* and by using qRT-PCR. The genes included in the analysis were chitin-binding receptor (*Bti9*, *SlLYK1*, *LYK4*), chitinase (*Chi1*), and β-1,3 glucanase (*PR2*).

Conclusion

Chitin fragments with an estimated degree of polymerization of less than 5 induced the overexpression of genes that encode for chitin receptor like protein kinase. These genes showed a high percentage of similarity to *AtCERK1* and *LYK4* reported in *Arabidopsis*, and highly similar to *Bti9* and *SlLYK1* reported in tomato plant. Likewise, the perception of fungal chitin fragments induced the overexpression of genes involved in the signaling mediated by JA and ET, activating the defense mechanism, which was reflected with the overexpression of genes that code for proteins related to pathogenesis.

The identification of different genes involved in the recognition of fungal chitin in tomato fruits represents an advance to understand the phenomena of fungal chitin perception in highly perishable fruits. However, more studies are required to generate information that allows proposing more effective control strategies that guarantee the develop of environmentally friendly alternatives for fruit safety.

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6. CONCLUSIONES

Los fragmentos de quitina de *Alternaria alternata* con un grado de polimerización estimado <5 monómeros y un grado de acetilación de 76.7 %, aplicados en frutos de tomate tipo bola durante 30 min, indujeron la sobreexpresión de genes que codifican a receptores que reconocen quitina. Estos genes mostraron un alto porcentaje de similitud con *AtCERK1* y *LYK4* reportados en *Arabidopsis*, y muy similares a *Bti9* y *SlLYK1* reportados en plantas de tomate. Asimismo, el reconocimiento de fragmentos de quitina fúngica indujo la sobreexpresión de genes implicados en la señalización mediada por AJ y ET, activando el mecanismo de defensa, lo que se reflejó en la sobreexpresión de genes que codifican para proteínas relacionadas con la patogénesis.

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