

Chitinases in plants and possible use as biomarkers for the design of biosensors in the detection of phytopathogenic fungi

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Abstract

Chitin is the most important biopolymer of the cell wall of fungi, which is degraded by the action of chitinases. Plants synthesize these enzymes to protect themselves from both abiotic and biotic factors, including phytopathogenic fungi, which remain in a dormant state until they find the right conditions to manifest themselves. For their identification, techniques based on biomarkers could be considered and devices that are fast, simple, specific and reliable could be created, such is the case of biosensors. The specificity of chitinases with chitin is widely known, so the identification of fungi could be carried out by means of a biosensor that includes chitinases. This manuscript reviewed information about the synthesis of chitinases in plants when subjected to stress, focusing on plant-pathogen pathosystems. The techniques and methods of identification of fungi are also mentioned, highlighting the use of biosensors. Finally, the use of chitinases as enzymatic biomarkers for their identification by means of a biosensor and their application in the control of phytopathogenic fungi is proposed.

Keywords: chitin, defense mechanisms, fungi.

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Chitin is the second most abundant biopolymer in nature after cellulose, which is synthesized by a large number of organisms and is found, among others, in the cuticle of insects, arachnids, exoskeletons of crustaceans and invertebrates such as mollusks, annelids and cephalopods. It is also a component of the cell wall of algae, nematode eggs and is a primary feature in the cell wall of fungi (Ramírez *et al.*, 2010; Castro *et al.*, 2011).

Chemically, chitin is composed of N-acetyl-D-glucosamine molecules bound with β -1,4 bonds, which requires for its synthesis the action of the chitin synthetase enzyme found in chitosomes. This enzyme uses UDP-N-acetylglucosamine as a substrate and a divalent cation, usually Mg, as a cofactor. Once the polymer forms at cytoplasmic sites, chitosomes translocate it across the membrane to the extracellular space where each polymer spontaneously assembles to form crystalline microfibrils that remain adjacent to the plasma membrane (Muzzarelli, 2011).

Chitin and its derivatives, such as chitosan and its N-acetylglucosamine oligosaccharides, have great chemical and thermal stability; however, they are susceptible to the action of chitinolytic enzymes (Ramírez *et al.*, 2010) and, according to cleavage patterns, chitinolytic enzymes are divided into: N-acetylglucosaminidases and chitinases. The first are glycoside hydrolases that catalyze the release of N-acetylglucosamine residues; whereas chitinases are the glycoside hydrolases that catalyze the hydrolysis of β -1,4 bonds in chitin and short-chain chito-oligomers, promoting their release with sizes ranging from 20 kDa to approximately 90 kDa (Seidl, 2008).

According to the place of cleavage of the chitin chain, chitinases can be classified into endochitinases or exochitinases, the former degrades anywhere in the chain, while exochitinases cut at the end of the chain, giving rise to chitobiose molecules (two units of N-acetylglucosamine) or N-acetylglucosamine molecules (Seidl, 2008). In addition, according to the amino acid sequence, catalysis mechanisms, substrate specificity and sensitivity to inhibitors, chitinases are phylogenetically classified into five classes: class I, II, III, IV and V. Classes III and V belong to the family 18 of glycoside hydrolases and are present in most organisms, such as fungi, bacteria, viruses, animals and higher plants; while the rest of the classes are part of the family 19 of glycosides hydrolases and are mainly present in some bacteria and higher plants such as corn and tomato, among others (Jashni *et al.*, 2015; Xu *et al.*, 2016).

The difference between class I and II is that the former have the chitin-binding domain in the N-terminal region of 40 amino acids rich in cysteine in class I; while class V chitinases contain two chitin-binding domains (Grover *et al.*, 2012). The present literature review mentions the action of chitinases as part of the defense system of plants and their activation in abiotic and biotic stresses, highlighting their synthesis in plants as a response to infection caused by phytopathogenic fungi; likewise, the different techniques for the identification of these microorganisms were mentioned, highlighting the use of biosensors. Likewise, the synthesis of chitinases was considered as a possible biomarker during the plant-fungus interaction in order to include them into the design of a biosensor.

Chitinases in plants

Plants, being sessile organisms, dedicate part of their energy to their defense against the adversities of the environment that surrounds them (Ortiz *et al.*, 2014). In this sense, the synthesis of chitinases in plants, which accumulate in the apoplast or in the vacuoles, is involved in the protection against biotic and abiotic factors, as well as in different physiological processes of the same plant (Sahai and Minocha, 1993). Each of the factors that cause the synthesis of these enzymes is briefly described below, focusing the research on biotic factors, in particular on phytopathogenic fungi.

Abiotic stress

Abiotic stress in plants encompasses all environmental conditions that reduce their correct yield and development; however, to reduce the negative effects of the environment around them, plants respond to this stress in different and complex ways (Cramer *et al.*, 2011). The main problems due to abiotic stress are due, among other things, to ultraviolet light, osmotic changes, temperature, drought and salinity. In all these cases, the synthesis of chitinases that act as a defense mechanism is stimulated (Grover, 2012; Xu *et al.*, 2016). Table 1 shows some examples of chitinase production due to some abiotic stressors.

Table 1. Summary of some scientific reports on the production of chitinases due to abiotic stress.

Agricultural product evaluated	Type of abiotic stress	Observations on chitinase production	Authors
<i>Arabidopsis</i>	Osmotic change	The overexpression of a chitinase present in <i>Arabidopsis</i> caused greater tolerance to osmotic stress induced by NaCl during seed germination and seedling growth.	Hong and Hwang (2006)
Saffron (<i>Crocus sativus</i>)	Wounds	Two hours after wounding the corms of saffron plants, the expression of a chitinase promoting the defense of the plant was observed.	Castillo and Gómez-Gómez (2009)
Chirimoya (<i>Annona cherimola</i>)	Cold	There was a prolonged increase in chitinase expression in chirimoya subjected to 6 °C during nine days of storage.	Goñi <i>et al.</i> (2009)
Apples (<i>Malus hupehensis</i>)	Phytohormones	An overexpression of three genes that encode chitinase was observed in apples after being treated with salicylic acid, methyl jasmonate and 1-aminocyclopropane-1-carboxylic acid for 48 h.	Zhang <i>et al.</i> (2010)

Agricultural product evaluated	Type of abiotic stress	Observations on chitinase production	Authors
Rice (<i>Oryza sativa</i>)	Heavy metals	The toxicity response to cadmium of two rice genotypes was evaluated: one susceptible and one tolerant to cadmium, finding an overexpression of chitinases in the roots of the tolerant rice genotype.	Cai <i>et al.</i> (2011)
Tea plant (<i>Camellia sinensis</i>)	Phytohormones	An increase in the transcription of a gene that encodes a chitinase present in tea plant leaves exposed to methyl jasmonate was reported.	Roy and Chakraborty (2012)

Chitinase activity during fungal pathogenesis

Among the multiple and complex defense pathways that plants have, there are reports of the action of chitinases in the protection against phytopathogenic fungi, taking the role of pathogenesis-related (PR) proteins and being an integral part of the mechanism of resistance to diseases that act as inducers, being categorized in the classes of PR-3, PR-4, PR-8 and PR-11 (Sánchez-García *et al.*, 2012; Xu *et al.*, 2016).

Phytopathogenic fungi contain chitin in their cell wall; so, the action of chitinases is directed towards this area to cause lysis of the fungus. Chitinases act in defense response to the attack of these by inhibiting the germination of spores, shortening the germ tubes and degrading the tips of the hyphae (Ntui *et al.*, 2011). When hyphae penetrate the intracellular space, apoplastic chitinases release inducing molecules that activate defense mechanisms and synthesize vacuolar chitinases and more apoplastic chitinases, improving infection signaling; meanwhile, when hyphae destroy the cell that causes the lysis, vacuolar chitinases are responsible for degrading the chitin chains of the invasive fungus, inhibiting their growth (Kasprzewska, 2003).

On this topic, Garg and Gupta (2010) tested the production of chitinases of bean (*Phaseolus aconitifolius*) plants by inoculating them with *Macrophomina phaseolina*, showing greater activity compared to untreated plants; the same results were obtained in *in vitro* and *in vivo* experiments. Similarly, Chaturika *et al.* (2011) discovered the antifungal activity of chitinase present in the aqueous phase of the latex of the mango (*Mangifera indica*) fruit, which digested the walls of the conidia of *Colletotrichum gloeosporioides*. Table 2 shows other examples of chitinase activity within the plant defense system.

Table 2. Summary of some reports on the antifungal activity of chitinases during the infection process.

Evaluated fungus	Main findings	Authors
<i>Phytophthora parasitica</i> var. <i>nicotianae</i>	The induction of chitinases in tobacco (<i>Nicotiana tabacum</i>) roots treated with chitosan was promoted, obtaining greater resistance to <i>P. parasitica</i> compared to the control.	Falcón <i>et al.</i> (2002)

Evaluated fungus	Main findings	Authors
<i>Sphaerotheca humuli</i>	A delay of the disease caused by <i>S. humuli</i> was observed in strawberry (<i>Fragaria x ananassa</i>) plants sprayed with a chitinase isolated and purified from the tuber of yam (<i>Dioscorea alata</i>).	Karasuda <i>et al.</i> (2003)
<i>Pyricularia grisea</i>	Higher chitinase production and lower incidence of <i>P. grisea</i> were observed in rice plants whose seeds were treated with chitosan, unlike the control.	Rodríguez-Pedroso <i>et al.</i> (2006)
<i>Colletotrichum falcatuma</i>	A greater presence of chitinases was reported in sugarcane (<i>Saccharum officinarum</i>) leaves resistant to <i>C. falcatuma</i> when subjected to the treatment of a glycoprotein isolated from the cell wall of the fungus for the induction of resistance proteins, compared to a susceptible variety.	Sundar <i>et al.</i> (2008)
<i>Mycosphaerella fijiensis</i>	Banana (<i>Musa</i> spp.) leaves resistant to <i>M. fijiensis</i> were inoculated with the fungus and an increase in chitinase activity was observed, unlike the susceptible variety.	Sánchez-García <i>et al.</i> (2012)
<i>Oidiopsis taurica</i>	An increase in chitinases was identified in roots and leaves of tomato (<i>Solanum lycopersicum</i>) var. Amalia, whose seeds were previously inoculated with the arbuscular mycorrhizal fungi <i>Glomus cubense</i> and <i>G. mosseae</i> , after being exposed to <i>O. taurica</i> .	Pérez <i>et al.</i> (2015)
<i>Colletotrichum falcatum</i>	Resistance to <i>C. falcatum</i> was reported in sugarcane plants with the addition of a chitinase-coding gene.	Tariq <i>et al.</i> (2018)

Likewise, the development of transgenic plants has been of great help in agriculture, favoring their yield and production by improving their specific genetic characteristics; therefore, one of the objectives of the genetic modification of plants is their protection against phytopathogenic organisms. Knowing of the action of chitinases against organisms that contain chitin in their morphology, the synthesis of a greater number of chitinases is the purpose of the genetic alteration of many plants (Grover, 2012).

However, despite the effectiveness of the plant defense system in preventing infection by phytopathogenic fungi through the synthesis of chitinases, there are some fungi such as *Fusarium verticillioides*, *F. oxysporum*, *Bipolaris zeicola*, *Stenocarpella maydis* and *Bipolaris zeicola* that can minimize this defense system through the synthesis of chitinase-modifying proteins (CMP) and proteases that degrade chitinases (Naumann, 2011; Jashni *et al.*, 2015). Table 3 shows some studies on genetically modified plants that overexpress some gene that encodes chitinases to give resistance against pathogens.

Table 3. Summary of some scientific reports on the antifungal activity of chitinases in transgenic plants.

Fungus	Main findings	Authors
<i>Venturia inaequalis</i>	The genes of <i>Trichoderma atroviride</i> that encode endochitinases or exochitinases were inserted into ‘Marshall’ and ‘McIntosh’ apples (<i>M. domestica</i>) individually and in combination. The resulting plants were selected to determine resistance to <i>V. inaequalis</i> , obtaining a decrease in the disease when both genes were present, compared to the expression of only one.	Bolar <i>et al.</i> (2001)
<i>Phoma tracheiphila</i> and <i>Botrytis cinerea</i>	The gene that encodes the chit42 endochitinase of <i>T. harzianum</i> was inserted into ‘Femminello siracusano’ lemon (<i>Citrus limon</i>) plants. They were tested against <i>P. tracheiphila</i> and <i>B. cinerea</i> , significantly inhibiting both pathogens, compared to plants without the gene.	Gentile <i>et al.</i> (2007)
<i>Alternaria solani</i>	Potato (<i>Solanum tuberosum</i>) plants were genetically transformed with the insertion of the gene that encodes the chitinase (ChiC) of the strain <i>Streptomyces griseus</i> HUT 6037. The plants showed high resistance against the fungus <i>A. solani</i> , compared to the control.	Khan <i>et al.</i> (2008)
<i>Fusarium graminearum</i>	Wheat (<i>Triticum aestivum</i>) plants were genetically modified with the insertion of the gene that encodes a class II chitinase in barley. The plants were inoculated with <i>F. graminearum</i> , which showed resistance to this pathogen.	Shin <i>et al.</i> (2008)
<i>Rhizoctonia solani</i>	Transgenic cotton (<i>Gossypium hirsutum</i>) plants that express the gene of an endochitinase of <i>T. virens</i> were confronted against <i>R. solani</i> , showing greater defense against this pathogen, compared to non-transgenic plants.	Kumar <i>et al.</i> (2009)
<i>Rhizoctonia solani</i>	The gen that encodes the chitinase (CHIT42) in the entomopathogenic fungus <i>Metarhizium anisopliae</i> was transferred by genetic modification to tobacco plants, which were consistently resistant to <i>R. solani</i> when compared to plants without the gene.	Kern <i>et al.</i> (2010)

Chitinases as biomarkers for the detection of fungi

Techniques for the detection of fungi

The attack by phytopathogenic fungi causes significant losses in fruits in pre- and post-harvest; therefore, a method that identifies the presence of these pathogens in a timely manner is necessary to apply some treatment that controls them. According to Ray *et al.* (2017), there are techniques that allow the detection of such pathogens, which are classified into two types: conventional and

emerging. The former refers to methods of culture by isolation and visual examination, where fungi or their spores are subjectively identified based on manuals according to their morphology; while emerging techniques are more specific techniques and are categorized into two methods: direct and indirect.

Indirect methods include techniques such as immunological methods and polymerase chain reaction (PCR) methods; however, despite being precise, they require time to give results; on the other hand, indirect methods use techniques based on biomarkers, which are in real time. Biomarker-based techniques focus on detecting the impact of the pathogen on plant physiology or post-contact stress and are categorized into spectroscopic and imaging techniques and volatile organic compound (VOC) detection techniques.

Among these methods are fluorescence spectroscopy, X-rays, nuclear magnetic resonance, visible infrared, thermography, gas chromatography-mass spectrometry, among others (Ray *et al.*, 2017). On the other hand, there are works that use techniques based on biological markers. An example of this is the work carried out by Crespo *et al.* (2008), who studied the composition of VOCs released by *Beauveria bassiana* in the presence of n-octacosane by means of gas chromatography-mass spectrometry (GC-MS). Also, Jones *et al.* (2011) used fluorescent stains based on calcofluor white to detect chitin in organisms of the genus *Cryptomycota*.

For their part, Berdugo *et al.* (2014) detected changes in the temperature of cucumber leaves related to transpiration during *Sphaerotheca fuliginease* infection by means of digital infrared thermography, differentiating healthy plants from diseased plants. In this regard, recent studies could consider biomarker-based techniques to create devices that are fast, simple, specific, and reliable for identifying phytopathogens of interest. Such is the case with biosensors.

Biosensors

A biosensor is a compact device that integrates a bioreceptor (nucleic acid, enzyme, protein, antibody, cell, etc.) associated with a transduction system that allows detecting the signal emitted by the interaction between the bioreceptor and the analyte of interest; the result of this interaction produces a variation of one or more physicochemical properties such as pH, color, heat, etc., which will be detected by the transducer and transformed into an electronic signal that indicates the presence of the analyte (González-Rumayor *et al.*, 2005).

Among the advantages of a biosensor are short analysis time, long lifetime, low production cost, automation, simple handling and portability, multi-analysis capacity and high selectivity, among others. According to their transduction system, biosensors are classified into piezoelectric, electrochemical, thermometric and optical (González-Rumayor *et al.*, 2005). The principle of some of them is explained in Table 4.

Table 4. Classification of biosensors according to their transduction system.

Biosensor	Principle
Electrochemical	It transforms the signal between the analyte and bioreceptor into an electrical signal; in turn, they are subdivided into conductometric, amperometric, potentiometric and impedimetric biosensors according to the detection of changes in these properties.
Optical	It measures variations in light properties such as absorption, scattering, luminescence, fluorescence, scattering or diffraction index during the interaction of the bioreceptor with the analyte.
Piezoelectric	It measures changes in mass produced by the antigen-antibody interaction by detecting variation in oscillation frequency.
Thermometric	They detect the heat generated in exothermic enzymatic reactions.

To date, the applications of biosensors in different fields are wide, for example, in the field of health for the detection of certain diseases and the care of the environment; likewise, the applications of a biosensor in the agri-food field are aimed at food safety and quality and control in industrial processes (González-Rumayor *et al.*, 2005; Castro-Ortíz *et al.*, 2007).

There is little information on biosensors that detect the presence of phytopathogenic fungi in food; however, some studies have been developed with this interest in recent years. Table 5 shows some studies of biosensors to determine the presence of fungi.

Table 5. Summary of some reports on the design of biosensors for the identification of phytopathogenic fungi.

Detection of:	Informative summary	Authors
<i>Aspergillus niger</i>	A prototype of a biosensor was made by synthesizing nanoparticles and nanolayers of copper oxide to detect <i>Aspergillus niger</i> by means of carbon dioxide produced by the fungus by means of an electrical resistance.	Etefagh <i>et al.</i> (2013)
1-octen-3-ol of the spores of <i>Aspergillus</i> and <i>Fusarium</i>	Biosensor based on an olfactory system integrated into platforms of carbon nanotubes to detect 1-octen-3-ol of <i>Aspergillus</i> and <i>Fusarium</i> spores present in grains.	Ahn <i>et al.</i> (2015)
<i>Pseudocercospora fijiensis</i>	Immunosensor to detect <i>P. fijiensis</i> in banana leaf extracts by immobilization, on a gold-coated chip, of a polyclonal antibody (anti-HF1), produced against HF1 in the cell wall of the fungus.	Luna-Moreno <i>et al.</i> (2019)
<i>Penicillium digitatum</i>	Biosensor to detect <i>P. digitatum</i> in oranges through the luminescent responses of bacteria to changes in the volatile organic compounds of the fruit during infection.	Chalupowicz <i>et al.</i> (2020)

Another form of classification of biosensors is according to the type of bioreceptor they contain (Monošíka *et al.*, 2012). Consequently, there can be enzymatic biosensors whose main characteristic is that they contain enzymes as a recognition element; once the enzymes bind at their active site with the analyte of interest, catalyzing the reaction, changes in temperature, mass, heat and electrical charges occur, which can be translated by a transduction system according to this reaction (Torres-Ramírez and Méndez-Albores, 2014). This type of biosensors can be used in complex mixtures, showing high selectivity and rapid response, managing to be used on more than one occasion (Serna-Cock *et al.*, 2009).

The implementation of chitinases for the design of biosensors that detect fungi has been very little explored; however, Pretty and Hodda (2018) designed an optical biosensor to detect the chitin of fungi. The authors used chitinases of *Vigna mungo* and the enzyme N-acetyl β glucosaminidase (NAGase) of *Canavalia ensiformis* for the detection of fungal chitin in wheat grains, concluding that where there was presence of fungi, the chitin content increased, so the chitinases degraded the biopolymer in their oligomers that increased absorbance.

Conclusions

The current development of new techniques for the detection of phytopathogenic fungi requires fast, accurate and reliable processes. In this sense, biosensors could be a viable option compared to other traditional techniques that, in addition to incorporating higher costs and/or people trained for such identification, are slow. Considering the activity of chitinases in plants as part of their defense system against the presence of phytopathogenic fungi, the design of a biosensor that incorporates these enzymes as biomarkers could generate valuable information in the agri-food field. To achieve this, it is first necessary to quantify the activity of chitinases in plants when attacked by fungi and then, design a device that integrates chitinases as a bioreceptor and that identifies the chitin of fungi as an analyte of interest present in a small sample of the plant; the interaction signal between the two can be translated by a suitable transduction system in real time.

However, it is necessary to consider those phytopathogenic fungi that degrade chitinases and that silence the synthesis of these enzymes; for this, it is necessary to detect chitinase-modifying proteins or specific proteases before obtaining the measurement of chitinases. Such a device would have the advantages of specificity and real-time accuracy that are required in the field of plant pathology.

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