Proteins and peptides for the control of phytopathogenic fungi

REVIEW

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ABSTRACT

Plants are exposed to a large number of pathogenic fungi. Although they do not have an immune system, plant have evolved a variety of potent defense mechanisms, including the synthesis of low molecular weight compounds, proteins, and peptides that have antifungal activity. These proteins appear to be involved in either constitutive or induced resistance to fungal attack. Generally, these proteins are not race- or species specific and have a broad spectrum of activity including: the inhibition of fungal cell wall synthesis or the disruption of cell wall structure, and/ or function as well as other disorders of the fungal membrane structure resulting in fungal cell lysis. This review is a compilation of the main pathogen defense related proteins and peptides.

Key words: antifungal proteins, pathogenesis-related proteins, RIP, LTP

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RESUMEN

Proteínas y péptidos para el control de hongos fitopatógenos. Las plantas tienen varios mecanismos de defensa inherentes como son la producción de proteínas relacionadas con la defensa, las cuales actúan para limitar la infección de los hongos patógenos. Estas proteínas están involucradas tanto en la resistencia constitutiva como inducida por el ataque del patógeno. Generalmente no son raza o especie específica y tienen un amplio espectro de actividad como inhibición de la síntesis de las paredes celulares del hongo o la ruptura de la estructura y/o función de la pared, otras perturban la estructura de membrana del hongo, resultando en la lisis de la célula del hongo. En este artículo se hace una recopilación de las principales proteínas y péptidos que tienen actividad antifúngica y sus modos de acción.

Palabras clave: proteínas antifúngicas, proteínas relacionadas con la patogénesis, RIP, LTP

*I*ntroduction

Plants have their own networks of defense against pathogens that include a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack. Not all pathogens can attack all plants and a single plant is not susceptible to the whole plethora of plant pathogenic fungi, viruses, bacteria or nematodes.

Recombinant DNA technology allows the enhancement of inherent plant responses against a pathogen by either using single dominant resistance genes not normally present in the susceptible plant [1] or by choosing plant genes that intensify or trigger the expressions of existing defense mechanisms [2]. The expression of cloned genes in transgenic plants has provided evidence on plant defense [3]. The genes encoding many antifungal proteins are currently being used to create genetically modified plants that have increased fungal resistance in the field [4]. Generally, these proteins are not race-or species-specific and have a broad spectrum of activity. The identification of such proteins would lead to the isolation of genes that have a great potential in developing transgenic plants with disease resistance traits [5]

These proteins appear to be involved in either constitutive or induced resistance to fungal attack. There are hundreds of antifungal peptides and proteins known, with more being discovered almost daily. Some of these proteins are: pathogenesis-related proteins, (PR), defensins, ribosome-inactivating proteins (RIP), lipid-transfer proteins (LTP), killer proteins, protease inhibitors, etc. These proteins have been named primarily on the basis of their mechanism of action, their structure or their similarity to a known "type" protein. Several classes of antifungal proteins involve the inhibition of fungal cell wall synthesis or cell wall structure and/or function disruption while others derange fungal membrane structure, resulting in fungal cell lysis. Additionally, the plants have others inducible defense mechanisms such as lignification, the production of peroxidase, salicylic acid, ethylene and hypersensitive reaction that act to limit pathogen infection [6].

*P*athogenesis-related proteins (PR proteins)

PR proteins are a group of diverse proteins whose accumulation is triggered by a pathogen attack, an abiotic stress, during hypersensitive response (HR) and also during systemic acquired resistance (SAR). These are therefore thought to have a role in natural defense or plant resistance to pathogens.

In a sense, PR proteins are an intersection point of various response networks by reacting with different inducers such as salicylic acid, jasmonic acid, systemin and ethylene. In theory, the constitutive expression of PR proteins, either singly or combined, may confer a decreased susceptibility to a specific group of pathogens [7].

These proteins have been classically divided into five groups, PR-1, PR- 2, PR-3, PR-4 and PR-5, based on serological and amino acid sequence analysis. Recently, another 6 groups of proteins have been suggested for their inclusion as PR proteins, forming a total of 11 groups [8]. Each of the five classical groups

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of PR proteins has two subclasses: a basic subclass found in the plant cell vacuole and an acidic subclass usually found in the extra cellular space [9]. Each group has members with antifungal activity, and cognates of most groups have been found in a diversity of other organisms. The antifungal action mechanisms of only the PR-2 and PR-3 groups of proteins have been clearly identified [10].

PR-1 proteins

These are proteins of low molecular weight (15-17 kDa). They have been found in rice, wheat, maize, tobacco, *Arabidopsis thaliana*, barley and many other plants [11-15]. They are homologous to the super family of cysteine-rich proteins. Although the exact mechanism of antifungal activity is not understood for plant PR-1 proteins, the constitutive expression of the PR1A gene in tobacco enhances plant resistance to *Peronospora tabacina* [16]. A PR-1 like protein, helothermine, from the Mexican banded lizard interacted with membrane-channel proteins of target cells, inhibiting the release of Ca^{2+} [17]. Whether the antifungal plant PR-1 proteins use this mechanism or not is unknown but suspected.

PR-2 proteins (β-glucanases)

PR-2 proteins have 1,3 β-endoglucanase activity *in vitro* and have been grouped into three classes on the basis of amino acid sequence analysis [18]. They have molecular masses between 33 and 36 kDa. PR-2 proteins have been found in a wide variety of plants, including tobacco, *Arabidopsis thaliana*, peas, sorghum, grains and fruits [19-22], and they are active *in vitro* at micro molar levels against a wide number of fungi. The antifungal activity of PR-2 has been convincingly demonstrated by a number of *in vitro* enzyme and whole-cell assays [23] as well as *in planta* using transgenic plants with an over expression of PR-2 proteins [24]. The antifungal activity of plant (1,3) β glucanases is thought to occur by PR-2 proteins hydrolyzing the structural (1,3) β glucan present in the fugal cell wall, particularly at the hyphal apex of filamentous molds where glucan is most highly exposed, resulting in a weak cell wall. This weakened cell wall results in cell lysis and cell death.

PR-3 proteins (chitinases)

PR-3 proteins have *in vitro* chitinase activity. Most PR-3 proteins have molecular masses of between 26 and 43 kDa and have been divided into five groups (Class I- Class V). Chitinases have been isolated from fungi [25], tobacco [26], cucumber, beans [27], grains [28] and other plants [29], and bacteria [30].They have potent antifungal activity against a wide variety of human and plant pathogens. By analogy with β-glucanases, the mode of action of PR-3 proteins is relatively straightforward: PR-3 proteins are endo-chitinases that cleave cell wall chitin polymers *in situ*, resulting in a weakened cell wall and rendering osmotically sensitive fungal cells [4]. A few transgenic crop species expressing chitinases have been evaluated in field trials and it was demonstrated that disease incidence was reduced [31]. There are a fewer examples of the expres-sion of chitinases in transgenic plants but the results have generally been similar to those for glucanase expression. The combined expression of chitinase and glucanase in transgenic carrot, tomato and tobacco was much more effective in disease prevention due to a number of pathogens than either one alone [32], con-firming the synergistic activity of these two enzymes reported from *in vitro* studies [31].

PR-4 proteins

PR-4 proteins are chitin-binding proteins, having molecular masses of 13-14 kDa and have been classified into two groups (Class I and Class II) [33]. PR-4 proteins have been isolated from potato, tobacco, barley, tomato, and many other plants [34-37]. Both classes of proteins have potent antifungal activity against a wide variety of pathogens. The antifungal activity of class I proteins is likely the result of protein binding to nascent fungal cell wall β-chitin. By mechanisms not yet understood this results in disrupted cell polarity, with a concomitant inhibition of growth [38].

PR-5 proteins

PR-5 proteins share a significant amino acid homology to thaumatin (a protein isolated from *Thaumatococcus danielli*) and are known as TL proteins. TL proteins have been isolated from A. *thaliana* [39], corn [28], soy beans, rice, wheat, tobacco [40], tomato [41] and many others [42]. The majority of PR-5 proteins have molecular masses of ~22 kDa. Osmotin is a basic 24 kDa protein belonging to this family having antifungal activity *in vitro* [43] and showing enhanced lytic activity when tested in combination with chitinase and β-glucanase [44]. Thaumatin-like proteins are also expressed in plants in response to a range of stress conditions and were demonstrated to have antifungal activity *in vitro* [40]. The exact mechanism of action of PR-5 proteins is not completely understood but it probably produces the lysis of the pathogen by permeabilizing the fungal cell wall [43].

Many PR proteins may be acting synergistically *in vivo* and may also show enhanced inhibition of fungal growth when tested in combinations *in vitro* [45].

As a general rule, the deployment of genetic engineering approaches that involve the expression of two or more antifungal gene products in a specific crop should provide more effective and broad-spectrum disease control than the single-gene strategy [31].

Defensins and thionins

Another group of antimicrobial activities are defensins and thionins. These are low molecular mass (~ 5 kDa), cysteine-rich peptides (45-54 amino acids in length) found in monocotyledonous and dicotyledonous plant species [46, 47], in mammals, fungi [48] and insects [49]. These peptides may exert antifungal activity by altering fungal membrane permeability and (or) inhibiting macromolecule biosynthesis. Plant and fungal defensines are positively charged, and in most cases contain four disulfide bonds that stabilized each protein in solution [50, 51]. In addition, most defensins are highly oligomeric *in situ* [52]. Defensins are classified into four groups (Group I-Group IV). In contrast to mammalian and insect defensins, plant defensins do not form channels either in artificial bilayers or in artificial liposomes [53] and they do not show significant hyphal permeabilization activity [54]. The over expression of

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defensins in transgenic plants was demonstrated with several different pathogens, including *Alternaria* [55], *Fusarium* [56] and they provided resistance to *Verticillium* in potatoes under field conditions [57]. The expression of the α-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens [58]. The over expression of an endogenous thionin enhances resistance in *Arabidopsis* against *Fusarium oxysporum* [56]. One of the best studied defensins is Rs-AFP2 (*Raphanus sativus* antifungal protein-2). Transgenic tobacco plants producing Rs-AFP-2 show enhanced resistance to the foliar pathogen *Alternaria longipes* [46]. Similarly, alfAFP (alfalfa antifungal peptide), a gene for cysteine-rich defensin from alfalfa seeds, when expressed under the control of a 35S promoter in transgenic potatoes imparted resistance to *Verticillium dahliae*, *Alternaria solani* y *Fusarium culmorum*, but not to Phytophtora *infestans* [57].

On the other hand, hevein, a non enzymatic chitin-binding protein of 43 amino acids from the latex of rubber trees, is cystein-rich and its precursor, a pre-protein homologous to the tobacco PR4 protein [45] was shown to have antifungal activity *in vitro* [59]. An agglutinin (UDA) isolated and characterized from *Urtica dioica* is another chitin-binding protein homologous to hevein and has two chitin-binding domains. Hevein and UDA are the only two chiting-binding plant lectins which have been shown to inhibit fungal growth *in vitro.* Transgenic tomato plants expressing the hevein gene showed fewer symptoms in slides of transgenic tomato fruits compared to controls when infected with *Trichoderma hamatum* [60]. The antifungal effects of hevein from rubber plants (*Hevea brasiliensis*) enhanced the resistance of transgenic plants to *Alternaria brassicae* [61]. However, the expression of *Amaranthus* hevein-type peptide and *Mirabilis* knottin-type peptide in transgenic tobacco did not enhance tolerance to *Alternaria longipes* or *Botrytis cinerea* [62].

Plant ribosome inactivating proteins (RIP)

These are plant enzymes that have 28 rRNA N-glycosidase activity, which, depending on their specificity, can inactivate conspecific or foreign ribosomes, thereby turning off protein synthesis. They remove an adenine residue from 28S rRNA. As a result, the 60S ribosomal subunit is not able to bind the elongation factor 2, producing the inhibition of protein elongation [63]. Plant RIPs inactivate foreign ribosomes of distantly related species and of other eukaryotes including fungi *in vitro* and *in vivo* [64]. RIPs have been classified into three groups and have been isolated from *Mirabilis expansa* [65], *Pisum sativum* [26], *Momardica charantia* [66], *Ricinus communis* [67], *Viscum album*, and many others [68], as well as from fungi, e.g., *Aspergillus giganteus* [69]. Unfortunately, the antifungal activities of only a few of the many RIPs have been described.

The most common cystolic type I RIP from the endosperm of cereal grains do not act on plant ribosomes but can affect foreign ribosomes, such as those of fungi [70]. The expression of barley seed RIP reduced the development of *Rhizoctonia solani* in transgenic tobacco [71], but had little effect on *Blumeria graminis* in transgenic wheat [72]. It has been demonstrated that the combined expression of chitinase and RIP in transgenic tobacco had a more inhibitory effect on *Rhizoctonia solana* than the individual proteins. Resistance levels improved when RIP was used combined with either PR2 or PR3 [24].

Recent studies with a type 2 RIP showed that the cell-binding B-chain (lectin) binds to fungal cells, forming a channel allowing the N-glycosidase A-chain to enter into the cells, resulting in RNA damage [73].

Lipid transfer proteins (LTP)

These proteins are so named because of their ability to stimulate the transfer of a broad range of lipids through the membrane *in vitro* and may also be involved in the secretion or deposition of extra cellular lipophilic materials such as cutin or wax. LTPs are small proteins (~ 8.7 kDa) of ~ 90 amino acids that are stabilized by four disulfide bonds with a central tunnel-like hydrophobic cavity. They have been isolated from a number of sources, including mammals, plants, fungi and bacteria [74-76]. LTPs are active *in vitro* against a number of bacteria and fungi although the mechanism of action is not known. These proteins may perhaps insert themselves into the fungal cell membrane, and the central hydrophobic cavity could form a pore, allowing the efflux of intracellular ions, leading to fungal cell death [4]. The facts show that several LTPs in maize, barley and pepper leaves were induced by pathogen infection [77], and some LTP isoforms in radish and sugar beet were found to inhibit the growth of bacterial and fungal pathogens *in vitro* [78]. Recent studies demonstrated that LTP110, a cDNA sequence encoding lipid transfer protein in rice seedlings, is able to inhibit the growth of *Pyricularia oryzae in vitro* [79].

Polygalacturonase inhibitor proteins (PGIPs)

These glycoproteins are present in the cell wall of many plants and can inhibit the activity of fungal endo-polygalacturonase [80]. PGIPs have been identified in extracts of several plants including pears, tomatoes and beans [81, 82]. It is presumed that polygalacturonases function in pathogen infection by facilitating host cell wall degradation and PGIPs interfere with this process [63]. The expression of PGIPs in transgenic plants led to contrasting results: in transgenic tomatoes expressing a bean PGIP, resistance to *Fusarium*, *Botrytis*, or *Alternaria* was not enhanced [80] while in transgenic tomatoes expressing a pear PGIP, there was a reduced colonization of *Botrytis cinerea*, which was observed as reduced number of lesions and a 25% reduction in the size of the lesions as well as a reduced post-harvest infection on fruits [83].

2S Storage albumins

Although 2S albumins are generally considered storage proteins, these are known to inhibit the growth of pathogenic fungi. Terras *et al.* [52] showed that a 14 kDa heterodimeric 2S albumin from *Brassicaceae* seeds inhibit fungal growth *in vitro.* Furthermore, thionin anti fungal activity was synergistically enhanced by either a small subunit (4 kDa) or a large subunit (10 kDa) of the radish 2S-albumin and also by three other 2S-albumin like proteins. These results suggest a dual role for 2S albumins, one as storage proteins and

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another in plant defense, although definite evidence for this can be only obtained producing transgenic plants with these genes [62].

Cyclophilin-like protein

Cyclophilins are a highly conserved group of proteins that are the intracellular receptors for cyclosporine. They have been found in a wide variety of organisms, including bacteria, plants, animals and fungi [84]. Recently a 1 kDa protein was isolated from mung bean (*Phaseolus mungo*) with an activity against *R. solani*, *F. oxysporum*, *B. cinerea* and *Coprinus comatus* [27]. This protein, called mungin, showed significant homology to cyclophilin and inhibited α and β-glucosidases *in vitro*. However, the antifungal mechanism of action of mungin is not known.

Protease inhibitors

Protein inhibitors of serine (e.g., trypsin and chymotrypsin) and cysteine proteases have emerged as a class of antifungal proteins that have a potent activity against plant and animal pathogens. Cysteine protease inhibitors have been isolated from a fourth group of cystatins, the phytocystatins [85, 86]. Although phytocystatins are active against plant pathogens such as *F. solani* and *Trichoderma* reesei [87], the mechanism of antifungal activity is not understood.

Serine protease inhibitors that have antifungal activity also have the interesting property of inhibiting α-amylase activity from insects but not from bacteria or mammalian sources [88]. These proteins are bifunctional, inhibiting enzymes as well as insect and fungal growth. Other bifunctional proteins from ragi (*Eleusine coracana*), wheat and barley have been isolated and characterized [89]. The mechanism of the antifungal activity of these proteins is not fully understood.

Non-plant antifungal proteins

Fungal growth is inhibited *in vitro* by cell wall degrading enzymes, mostly chitinases, from various fungi. Some of these chitinases show synergy with PR5 proteins or other membrane affecting compounds and other fungal cell wall hydrolases [44]. An exochitinase gene from bacterium *Serretia marcescens*, when expressed in transgenic tobacco, renders the host plants less susceptible to *R. solani* [90]. A fungal chitinase gene from *Rhizopus oligosporus* confers antifungal activity to transgenic tobacco [91].

Killer proteins (killer toxins)

A number of yeasts secreted proteins are lethal to sensitive fungal cells. These proteins, called killer proteins or killer toxins, are encoded either by double-stranded RNA, linear double-stranded plasmid DNA, or nuclear genes [92]. Fungal cells secreting a killer toxin are resistant to their own toxin but are sensitive to other toxins. Over 20 individual killer toxins have been identified, varying in molecular mass from 10.7 to 156.5 kDa [93, 94]. The killer toxins have a broad, potent antifungal activity against a number of human and plant pathogens. Although they vary in their mechanisms of action, the first step of killer proteins activity involves binding the protein to specific cell surface receptors. Once bound, killer proteins are internalized and can disrupt cell wall synthesis, DNA synthesis, and K+ channel activity, inhibit (1,3) β-glucan synthesis, or arrest the cell cycle.

Concluding

Plants have several inherent inducible defense mechanisms such as: PR proteins, defensins, thionins, RIPs, LTPs and many others, that limit pathogen infection and are produced to protect them against microbial attack. The mechanisms of action of these proteins are as varied as their sources and include fungal cell wall polymer degradation, membrane channel and pore formation, damage to cellular ribosomes, inhibition of DNA synthesis, and inhibition of the cell cycle.

Recombinant DNA technology allows the deployment of genetic engineering approaches that involve the expression of two or more antifungal gene products in a specific crop that should provide more effective and a broader-spectrum in disease control than the single-gene strategy. The genes encoding many antifungal proteins are currently being used by agribusiness to create genetically modified plants that have increased fungal resistance in the field.

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