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Antifungal Proteins: Potent Candidate for Inhibition of Pathogenic Fungi

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Abstract: Fungi are far more complex organisms than viruses or bacteria and can develop numerous diseases in plants that cause loss of big portion of the crop every year. Another important aspect is that many filamentous fungi are destructive human pathogens and are thus responsible for enormous diseases in humans. A series of molecules with antifungal activity against different strains of fungi have been found in plants, which are of great importance to humans. Plants have developed various mechanisms to defend themselves against these fungi which include the production of low molecular weight secondary metabolites, proteins and peptides having antifungal activity. In this review, brief information like structure, source, mode of action of defense mechanism and their promising contribution in the field of medicine and agriculture is discussed. These molecules may be used directly or considered as a precursor for developing molecules with better therapeutic values. This review attempts to summarize the current status of important antifungal proteins from various natural occurring sources like plants, bacteria and insects.

Keywords: Pathogenesis-related proteins, β-glucanase, chitinases, thaumatin like protein.

INTRODUCTION

Fungi are an extremely diverse group of organisms, with about 250,000 species widely distributed in every ecosystem [1, 2]. More than 300 fungal species are reported to be origins of major diseases in plants and human beings. They are capable at colonizing and using plants, humans, and animals as substrates [3]. In the developed countries, these infections predominantly occur in the context of increasingly aggressive immunosuppressive therapies. The overall mortality for invasive diseases caused by *Candida* spp. and *Aspergillus* spp. is 30–50%. In the developing countries, one million cases of cryptococcal disease per year, resulting in 675, 000 deaths have been reported [4]. Allergic fungal syndromes are increasingly recognized [5]. Different kinds of mycoses, especially invasive, have become an important public health problem as their incidence has increased dramatically in the last decades in relation to AIDS, hematological malignancies, transplant recipients and other immunosuppressed individuals [1]. The filamentous fungus *Aspergillus fumigates,* the dimorphic yeast *Cryptococcus neoformans* and *Candida albicans* are the three predominant causative agents of human diseases [6, 7].

Some of the most common plant-pathogenic fungi belong to the genera *Alternaria, Botrytis, Cochliobolus, Geotrichum, Penicillium, Sclerotina, Fusarium* species and *Magnaporthe grisea* [8, 9, 10]. These fungi cause considerable loss of crop yields worldwide. Moreover, many potentially human-pathogenic fungi and the yeast *Saccharomyces cerevisiae* have their natural habitats in the environment, including plants and food items [11].

Currently, fungal infections are increasingly common and, in certain vulnerable patients, can be serious and even life threatening, therefore, lead to an increase demand for antifungal drugs. Still, antifungal treatment is limited to only a small number of drugs such as azoles, echinocandins and polyenes. They frequently interact infection unfavorably with other medications which leads to side effects and thus toxic to human beings [6, 12]. So, there is a substantial demand for new molecules with extensive antifungal activity and low toxicity to plants, animal and human being as compared to synthetic compounds or drugs.

Plants have evolved an array of defense mechanisms to mediate fungal disease resistance. Plant-produced proteins and peptides have been identified, few are commercially used. Micro-organisms have also been an important source of biologically active molecules [13] like bacteria [14], fungi [15]. Similarly, insects [16], mollusks [17] and mammals [18] synthesize a number of proteins and peptides that are antifungal. These proteins appear to be involved in either constitutive features of the structure or induced resistance against phytopathogenic fungi. Plants should be an excellent source of potent antifungal, since they are exposed to a wide array of phytopathogenic fungi present in their environment and have led to develop antifungal molecule to survive. Among the antifungal compounds produced by plants, insects and bacteria small-sized antifungal protein and peptides with suppressive effects on fungal growth have attracted considerable interest [14, 16, 19]. Despite the existence of defense mechanisms, plants are exposed to attack by fungi and bacteria. Fungi attack leaves, stems, roots, fruits, and flowers before and after harvest.

Antifungal proteins have been isolated from a variety of plants [19, 20, 21], animals [22, 23], bacteria [14] and fungi [15]. They serve to protect these organisms against fungal invasion. Antifungal proteins can be divided, according to their structures, and/or functions, into different types comprising thaumatin-like proteins, protease inhibitors, chitinases, glucanases, ribosome inactivating proteins, em-

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bryo-abundant proteins, cyclophilin-like proteins, ribonucleases, arginine- and glutamate-rich proteins, peroxidases, and novel proteins and peptides. A great variety of antifungal proteins display a wide range of molecular weights, ranging from a few thousands [26, 27] to about 60 kDa [28, 29]. Below is a brief review of these antifungal proteins, current knowledge on structural features, antifungal activity against phytopathogenic fungi. It is expected that antifungal proteins will be important determinants in biotechnology approaches to improve the current repertoire to combat the fungal pathogens.

FUNGAL CELL WALL

The fungal cell wall, a structure with no mammalian counterpart, presents an attractive therapeutic target. It protects the organisms against a hostile environment and relays signals for invasion and infection of a likely plant, animal, or human host. Fungal cells are covered on the surface by rigid cell walls which determine the shape of the cell and protect the protoplast against adverse effects of the environment. The shape and integrity of the fungus are depended upon the mechanical strength of the cell wall, which performs a wide range of essential roles during the interaction of the fungus with its environment [30]. Fungal cell wall accounts for ~20 -30% of the total dry weight of fungal cell. The fungal wall is a complex structure composed typically of chitin, 1, 3-β- and 1, 6-β-glucan, mannan and proteins. The fungal wall affords a clear and distinct difference between fungi and their plant and animal hosts, providing an experimental target for antifungal antibiotics. Several classes of antifungal proteins have been shown to involve in inhibition of the synthesis of the fungal cell wall or disturb structure and function resulting in fungal cell lysis. One of them is, Chitinases, which catalyze the degradation (lysis) of cell wall chitin molecules. This process is a prerequisite for the onset of hypha branching. Chitinase had been reported to be produced by a large number of bacteria [31]. Apart from the exoenzymes chitinases (glycoside hydrolases), they form chitobiases that degrade chitin to N-acetyl-D-glucosamine. The fungal growth is affected by chitin-binding proteins chitinases and glucanases.

PATHOGENESIS-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 great role in natural defense or plant resistance to pathogens. PR proteins were first described by van Loon and van Kammen (1970) as components of the hypersensitive response in leaves of tobacco plants exposed to tobacco mosaic virus (TMV). The change in ion fluxes across the plant cell membrane, generation of active oxygen species, changes in the phosphorylation state of regulatory proteins and tran-

Table 1. Classification of Pathogenesis-Related Proteins (PRs)

Family	Member type	Properties	References
PR-1	Tobacco PR-1a	Antifungal (14-17Kda)	Niderman *et al*. 1995
PR-2	Tobacco PR-2	β- 1, 3-Glucanase (25-35KDa)	Leubner-Metzger and Meins 1999
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII (30KDa)	Gomez *et al*. 2002
PR-4	Tobacco R	Chitinase type I, II (13-19KDa)	Caporale *et al*. 2004
PR-5	Tobacco S	Thaumatin-like proteins,	Reiss *et al*. 2006
PR-6	Tomato inhibitor I	Protease inhibitor (6-13kDa)	Mosolov and Valueva 2008
PR-7	Tomato 69	Endoproteinase	Vero and Conejero. 1988; Tornero *et al*. 1996
PR-8	Cucumber chitinase	Chitinase type III	Kasprzewska 2003
PR-9	Tobacco	Peroxidase	Almagro *et al*. 2009
PR-10	Parsley PR-1	'Ribonuclease- like'	Somssich *et al*. 1986; Liu and Ekramoddoullah 2006
PR-11	Chitinase type V	Chitinase type I	Gomez *et al*. 2002
PR-12	Radish Ps-AFP 3	Defensin	Terras *et al*. 1992; Portieles *et al*. 2006
PR-13	Arabidopsis THI2-1	Thionins	Epple *et al*. 1995; Stec 2006
PR-14	Barley TLP 4	Lipid transfer protein (ns-LTP)	Garcia-Olmedo *et al*. 1995
PR-15	Barley OxOa (germin)	Oxalate oxidase	Zhang *et al*. 1995; Lane *et al*. 2002
PR-16	Barley OxOLP	'Oxalate oxidase-like'	Wei *et al*. 1998
PR-17	Tobacco PRp27	Unknown	Okushima *et al*. 2000; Christensen *et al*. 2002

scriptional activation of plant defense systems close in cell death at the site of infection, local accumulation of phytoalexins and cell wall rigidification as a result of callose, lignin and suberin deposition [32]. In addition, various novel proteins are induced which are collectively referred to as "pathogenesis-related proteins" (PRs). The inducible PR proteins, elicited by environmental and developmental stimuli, are also present constitutively in different plant organs. PRs accumulate locally at the site of infection, and systematically in the whole plant as part of systemic-acquired resistance against further infection [33, 34]. Induction of PRs has been found in many plant species belonging to various families [35], based on distribution of amino acid sequence, serological relationship [33] and antifungal activity [2]. Recently these PRs have been classified into 17 classes of families [36]. Within each PRs family, there are several classes comprising different isoforms, either basic protein are constitutively expressed in certain organs or acidic proteins may be induced only in response to certain stress signals [37]. Each group has members with antifungal activity, and cognates of most groups have been found in a diversity of other organisms. PRs represent a non-homogeneous group in respect to molecular weight (Mw), isoelectric point (pI) and immunological cross-reactivity; they share common structural characteristics [34, 36]. They are stable in low pH, are resistant to protease activity, are monomers of low molecular weight, and show apoplastic location [38]. The stability in low pH and resistance to proteases are characteristics of food allergens. Plant-derived allergens have been identified with sequence similarity to PR-2, PR-3, PR-4, PR-5, PR-6, PR-8, PR-9, PR-10, PR-14 [39], PR-15 and PR-16 [40] (Table **1**).

PRs exhibit broad diversity of mechanisms of action on microorganisms and pests. PRs activity is often specific towards a particular microbial species, i.e., recognizing specific structures on the microbial cell surface before damage to the membrane occurs [41]. They have typical physicochemical properties that enable them to resist acidic pH and proteolytic cleavage, allowing them to survive the harsh environments of the vascular compartment or cell wall or intracellular spaces [42]. The significance of PR proteins lies in their strong antifungal and antimicrobial activity. Several glucanases and chitinases have been shown to have antifungal properties, although these appear to be restricted to certain fungi [43]. Antifungal PR proteins are of great biotechnological interest because of their potential use as food and seed preservative agents and for engineering plants for resistance to phytopathogenic fungi. Members of the 5 major PR families (PR-1 through PR-5) have been shown to have antifungal activities and some related groups have been found in diversity of other organisms (Table **2**).

DIFFERENT CLASSES OF ANTIFUNGAL PROTEIN

Pathogenesis-Related (PR-1 Proteins)

The first PR- 1 protein was discovered in1970. The PR-1 groups were first isolated from tobacco leaves after infection by the tobacco mosaic virus [44, 45]. These proteins are accumulated to high levels after pathogen infection and are antifungal both in *planta* (transgenic plants over expressing tobacco PR-1) and *in vitro* [2]. A number of PR-1 proteins have been identified in *Arabidopsis,* barley, tobacco, rice, pepper, tomato, wheat and maize [46]. They are homologous to the super family of cysteine-rich basic proteins and its molecular weight of 15 to 17 KDa. PR-1 family members are also present in non-plant systems. A novel PR-1 family member localizes to the cytosolic site of the endomembrane system in mammalian cells [47]. Members of the PR-l protein family from tobacco and tomato have been shown to expressed constitutively the PR-1a exhibited increased tolerance and have *in vitro* antifungal activities against oomycetes fungi like *Peronospora tabacina* and *Phytophthora parasitica* var.*niocotianae* [33, 48]. PR-1-like protein, helothermine, from the Mexican banded lizard have been found to be interacting with the membrane-channel proteins of target cells, inhibiting the release of Ca_2^+ [1, 49]. However, the exact mechanism by which PR-1 proteins mediate disease resistance is still unknown. The fungicidal activity of the P14 protein [50] tomato and tobacco proteins related to the PR-1 family. They exhibited differential antifungal activity *in vitro* and *in vivo* (leaf disc assay) toward *P. infestans.* Tomato P14c exhibited the strongest antifungal activity against *P.infestans.*

Glucanases (PR-2 Proteins)

PR-2 proteins consist of a β-1, 3-glucanases (β-1, 3-Gs) comprises of large and highly complex gene families involved in pathogen defense as well as a wide range of normal developmental processes. The endo-β-1, 3-glucanases are abundant proteins widely distributed in seed-plant species [51, 52]. Glucans are a major component of the fungal cell wall. These endo-glucanases catalyzes several cleavages like α1-3, β1-4 andβ1-6 D-Glucosidic linkages in Glucans, So that cell lysis and cell death occur as a result of hydrolysis of glucans. These are therefore potent antifungal proteins. They are found in all organisms like plants, bacteria, fungi and invertebrates. PR-2 proteins are grouped into 3 classes: Class1 glucanases are basic proteins of 34kDa and are found in the plant vacuoles. They are synthesized as preproproteins and have no enzymatic activity until they are processed [53, 54]. Classes II and III include acidic, extracellular proteins of about 36 kDa. They are found in interstitial tissues of barley leaves that contain Pr-32 which is having antifungal activity. In tobacco, specific β-1, 3-glucanases from alfalfa, barley, tobacco and soybean have been shown to suppress fungal diseases. Overexpression of glucanases from soybean has been demonstrated to enhance protection of potato from *P. infestans* and kiwi from *B. cinereu* [55]. Whereas a glucanase of potato was reported to increase resistance in flax against F. *oxysporum* and *F. culmorum* [56]. In combination of β-1, 3-glucanases and chitinases, they act as synergistically and show inhibitory effects against a wide range of fungi including human and plant pathogens like *R.solani, C.albicans* and *A.fumigatus* [2].

Chitinases (PR-3 Proteins)

Chitinases are enzymes that hydrolyze the N-acetylglucosamine polymer, chitin, and these are present in plant tissues of a broad range of crop and noncrop species [57]. Chitinase can be isolated from chickpea [58], cucumber, barley [59], tobacco [60], black turtle bean [61], tomato [62] and grapes [63]. Chitinase having molecular mass in the

Table 2. Summary of the current knowledge regarding the source,mechanisms of action and antifungal activity of antifungal protein from various sources

Protein	Sources	Mode of action	Target organisms	*In vitro* MIC(IC 50)	Reference
Plants					
TLP F1 (PR-5)	*Oryza.sativa*	lysis	*F. graminearum, B. cinerea*	500 µg/ml	Jayaraj *et al*. 2003
Zeamatin	*Zea mays*	lysis	*C.albicans*	0.5 µg/ml	Robert and Selitrennikoff 1990
Mammalian					
Gallinacin-1	chicken	lysis	*C.albicans*	25.0 µg/ml	Harwig *et al*. 1994
NP-1	Rabbit granulocytes	lysis	*C.neoformans*	3.75-15.0 µg/ml	Alcouloumbre *et al*. 1993
Insects					
Drosomycin	D.melanogaster	lysis	*F. oxysporum*	5.9-12.3 µg/ml	Michaut *et al.* 1996
Cecropins B	H.cecropia	lysis	*A.fumigatus*	9.5 µg/ml	De Lucca *et al.* 1998
Camelyn "M"	Honey bee	-	C.albicans	0.012 µg/ml	Maglakelidze *et al.* 2011
Bacteria					
CB-1	*B.licheniformis*	chitin binding	*F.oxysporum*	50.0 µg/ml	Oita *et al*. 1996
Serratia marcescens NPLR1	*S.marcescens*	-	*A.niger*	1000 µg/ml	Sumathi *et al.* 2011
Bacillomycin F	*Bacillus subtilis*	Lysis	*A.niger*	40.0 µg/ml	Mhammedi *et al*. 1982
Fungi					
WF11899 A	Coleophoma Empetri	Glucan synthesis	*C. albicans*	0.16 µg/ml	Anthony *et al*. 2000
Mulundocandins	Aspergillus syndowi, var. mulundensis.	-	*A. niger*	31.3 µg/ml	Roy *et al*. 1987

range of 26 kDa and 43 kDa and have been divided into five main classes (I to V). Class I chitinases are basic proteins with an N-terminal cysteine rich domain which having molecular mass of about 32KDa and a highly conserved main structure that contains the catalytic domain that function as chitin-binding hevein-like domain.

Class II chitinases lack the N-terminal cysteine-rich domain but have high amino acid sequence identity to the catalytic domain of class I chitinases. Class III chitinases have no sequence similarity to class I or II enzymes and also possess lysozyme activity [64] and its molecular masses about 27 to 28KDa [2]. Class IV chitinases show 41 to 47% sequence identity to the main structure of class I chitinases. These chitinases also have a cysteine-rich domain that resembles that of the class I chitinases but is significantly smaller due to one and three deletions in the cysteine- rich and catalytic domains, respectively [2, 65, 66]. Class V chitinase sequences are not similar to those of members of the other classes but have homology with bacterial exochitinases and the encoded proteins display antifungal activity [67] Chitinases have significant antifungal activities against plant pathogenic fungi and human pathogens like *Alternaria* sp. For grain discoloration of rice, *Bipolaris oryzae* for brown spot of rice, *Botrytis cinerea* for blight of Tobacco, *Curvularia lunata* for leaf spot of clover, *Fusarium oxysporum*, *F. udum*, *Mycosphaerella arachidicola*, *Pestalotia theae* for leaf spot of tea and *Rhizoctonia solani* for sheath blight of rice [58, 59, 61] and *Coprinus comatus.*

Chitin Binding Protein (CBP PR-4 Proteins)

PR-4 proteins have been isolated from many groups of plants like sugar beet, tomato, potato, pepper, hortensia and barley and bacteria like *Streptomyces tendae* [14, 68, 69, 70]. CBP PR-4 proteins of molecular weights between 13 and 14.5 kDa. These proteins are clearly distinguishable from other PR proteins but are serologically similar to tomato protein P2, which is induced by *Cladosporium fulvum* infection [71]. These are classified into two groups {Class 1, Class 2} [54, 72, 73]. Class 1 CBPs having N-terminal chitin -binding domain similar to a domain hevein, a protein isolated from rubber latex and also belong to a superfamily of chitin-binding lectins. Class 2 protein lacks the chitin -binding domain as well as hevein domain also. Both classes of proteins have potent antifungal activity against a variety of Plant and human fungal pathogens like *Aspergillus* species, *Cercospora beticola*, *F.oxysporum, N.crassa* and *Xanthomonas campestris.*

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 result in disrupted cell polarity, with a concomitant inhibition of growth [14]. *In vitro* antifungal assays demonstrated that CDP 20 has antifungal properties against *Trichoderma viride* and *F. solani* by causing lysis of germ tubes and growth inhibition. In addition, CBP 20 interacted synergistically with a tobacco class I chitinase against *F.solani* and with a tobacco class I P-1, 3-glucanase against *F. solani* and *Alternaria radicina* [74].

Recently, the conserved region, which is common to both classes I and 2 is the Barwin domain, shows inhibitory activity on the growth of *T. harzianum* [75] and rice PR-4 shows antifungal activity *in vitro* against the sheath blight fungus *R.solani* [76]. Binding of the PR-4 members with the chitin-binding domain to chitin in the developing fungal cell walls might result in fungal growth retardation. Ribonuclease activity of some cereal seed PR-4 has been observed and the effect on the invading microorganisms explained alternatively as inhibition of the translation process [77].

Thaumatin-like Proteins (TLP PR-5 Proteins)

The Members of the PR-5 group of PR proteins are called thaumatin-like proteins (TLPs) because their amino acid sequences are homologous to that of thaumatin, a sweet-tasting protein from the West African shrub *Thaumatococcus danielli* [78]. Most of the TLPs have a molecular weight in the range of 18 kDa to 25 kDa and have a pH in the range from 4.5 to 5.5 [79, 80]. TLPs have been isolated from barley, kiwifruit, maize, douglas-firs, tobacco, tomato, wheat [79, 80, 81, and 82] corn [83] and *A. thaliana* [84]. TLP PR-5 proteins belong to a larger family of proteins that includes permatins from monocot grains and can permeabilize fungal membranes [85].

PR-5 proteins can be categorized into 3 subclasses based on pH: acidic, neutral, and very basic member [86], with an extracellular and vacuolar localization. There are several TLPs which play a significant role in protection against fungal attacks during dormancy and germination.A number of PR-5 proteins bind 1, 3β-glucan and have detectable *in vitro* 1, 3β-glucanase activity [87]. Zeamatin is a protein isolated from corn seeds, have been described to inhibit mammalian trypsin and insect α-amylase [88]. Osmotin which is inducible by pathogens and osmotic stress, and its homologs in tomato and potato have *in vitro* anti oomycete activity against *P. infestans*, and transgenic tobacco and potato plants have enhanced resistance against this pathogen but not against P. *parasitica f.sp. nicotianae*. Osmotin and zeamatin were both effective against the fungal pathogens like *Candida albicans, Neurospora* crassa and *Trichoderma viride* [89]. Linusitin is a 25-kDa TLP isolated from flax seeds. Linustin shows antifungal activity against *Alternaria alternata* by the mechanism of membrane permeabilization [90]. Barley seeds TLPs contain the antifungal proteins R and S, these are highly basic protein isolated from malting barley grain which are homologous to thaumatin and other PR-5 family proteins [91, 92] Barley seed TLPs inhibit growth of fungal pathogens *T. viride, Candida albicans* [92, 93], *Micrococcus lysodeikticus* and *F. sporotrichioides* [94].

Protease Inhibitors (PIs PR-6 Proteins)

Proteins inhibitors of serine (e.g trypsin and chymotrypsin) and cysteins proteases have emerged as a class of antifungal proteins that have potent activity against plant and animal pathogens [195]. It comprises a large and diverse group of proteins able to inhibit insects and other invertebrate pests (nematodes and mollusks), as well as animal and bird gut digestive enzymes [95, 96, 97] and fungal proteases [98, 99]. Antifungal cysteine protease inhibitors (cystatins) have been isolated from a fourth group of cystatins, phytocystatins and many other plants, although phytocystatins are active against plant pathogens such as *F. solani* and *Trichoderma reesei* [100, 101, 102] and its mechanism of antifungal activity is not yet understood.

PIs can be divided into two classes, based on the protease inhibition mechanism: inhibitors employing standard mechanisms (Mm up to 22 kDa) and the serpins (serine proteinase inhibitors) (Mm ~ 40 kDa) employing a so-called suicidal mechanism [103,104]. Currently there is a wide array of PIs found in storage seed like cereal seeds. Pls plays a major role in the inhibition of insect gut enzymes, they are also able to confer resistance against fungal diseases such as *Fusarium* head blight its one of the most devastating diseases of barley [36].

Peroxidases (PR-9)

Peroxidases are involved in detoxification of reactive oxygen radicals, which can damage of DNA and proteins, and severely compromise the function of the membrane. These heme or non-heme-containing enzymes that catalyze the oxidation of a variety of molecules using H_2O_2 as an electron acceptor. Heme peroxidases consist of the animal peroxidase superfamily, the non-animal (plants, fungi, and bacteria) peroxidase super family, catalases, di-heme cytochrome C peroxidases, Dyp-type peroxidases, and heme haloperoxidases [105]. In plant, Peroxidases are heme containing glycoproteins that participate in a great number of physiological processes, biosynthesis of ethylene, lignifications and suberinization of host cells during defense against pathogens, wounding auxin metabolism, and stress response [106, 107, 108]. In animals, peroxidase enzymes are involved in phagocytosis and immune cell function, cell adhesion, antioxidant function and the oxidative polymerization of hydroquinones to melanin [109-114]. The protein named Limlin has been isolated from *Phaseolus limensis* showed antifungal activity on fungi *F. solani*, *M. arachidicola* and *P. aphanidermatum* [115]. Direct antifungal activity of peroxidases in the absence of H_2O_2 has also been reported [116]. Seed peroxidases participate in the lignification of plant cell walls, which help to restrict the movement of the invading fungi [117, 118].

Ribonucleases-like Proteins (PR-10)

PR-10 is a family of small homologous, primarily acidic molecules present in a variety of angiosperms [190]. Several members of this group possess ribonuclease properties and are antifungal. It is an intracellular protein (17.3 kDa) located in the cytosol and is known to inhibit hyphal extension of *Phytophthora capsici*, possibly by inhibition of translation activity [191,192]. It shares similarity with the allergen Bet v 1, known to have ribonuclease activity in white birch. They were first identified in parsley *Petroselinum crispum* [120] and in major latex proteins found in Arabidopsis, Bell pep-

per, Mellon, Strawberry, and Tobacco [193,190]. The TcPR-10 protein has a promising biotechnological potential to act as a ribonuclease and presents antifungal activity against *M. perniciosa*, the causative agent of witches' broom disease, which is one of the most devastating diseases of cocoa plants [119]. Ocatin is a 18-kDa storage protein in the Andean tuber, oca (*Oxalis tuberosa*), and is classified as an antifungal of the PR-10 group. This protein constitutes between 40% and 60% of the total soluble oca tuber proteins and, *in vitro*, inhibits the growth of several phytopathogenic fungi, including *F. oxysporum* and *Rhizoctonia solani* [194].

Defensins (PR-12)

Defensins are a diverse group of low – molecular mass (5kDa), cysteine- rich peptides [45-54] amino acids in length) found in monocotyledonous and dicotyledonous plant species [198, 206] and show a broad spectrum of biological function [121, 122]. Distantly related peptides are also found in mammals, fungi [196] and insects [197]. These peptides may exert antifungal activity by altering fungal membrane permeability and (or) inhibiting macromolecule biosynthesis. Plant and fungal defensins are positively charged, and in most cases contain four disulfide bonds that stabilized each protein in solution [199, 200]. They are able to inhibit the growth of Gram-positive and Gram-negative bacteria, as well as fungal pathogens, with either morphogenic or non-morphogenic effects. Defensins inhibit insect digestive enzymes, proteinases and/or α-amylases, protein synthesis and the activity of ion channels [124, 125]. Defensins inhibit plant and human fungal pathogens, including *Alternaria, Fusarium, Candida* and *Aspergillus* species, and are employed as novel leads in antifungal therapeutics [201].

Thionins (PR-13)

Thionins consists of small basic proteins with approximately 45-47 residues long, in which 4-8 of these are cysteine residues that form disulfide bonds [126, 127]. They appear to play diverse role in nature, showing antifungal and /or antibacterial activity [128, 129]. They have the ability to inhibit mammalian cell growth by membrane permeabilization [130] and the capability of inhibit insects α-amylases and proteinases. Antifungal activity may also be based on the ability of the thionins to bind to the cell wall. Barley and wheat α-thionins bind polysaccharides containing chitin and β-glucan [131]. Thionins might be able to form ion channels in cell membranes; the highly conserved Tyr-13 might be involved in pore formation [132] and thus confer the antifungal activity.

Lipid –transfer Proteins (LTPs PR-14)

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. These Proteins are isolated from various sources like plants, mammals, fungi and bacteria [133]. LTPs are divided into two groups: LTP1 with molecular mass 9KDa and LTP2 with 7kDa [134]. LTPs have been shown to be active *in vitro* against a number of bacteria and fungi, although the mechanism of action is not known. All of these proteins possessed *in vitro* growth inhibitory activity against *F. solani.* Furthermore, these cereal ns-LTPs combined with a thionin had synergistic antifungal effects against the F. *solani* pathogen. 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 [2]. The facts show that several LTPs in maize, barley and pepper leaves were induced by pathogen infection [135]. LTP110, a lipid transfer protein from rice was cloned, expressed and tested *in vitro* against rice pathogens, *Pyricularia oryzae* and *Xanthomonas oryzae*. LTP110 was able to inhibit the germination of *P. oryzae* spores, but only slightly inhibited the growth of *Xanthomonas* [136].

PR-15 (Oxalate Oxidase)

Recently introduced families of PRs, oxalate oxidase (OXO) or germins (PR-15)368, oxalate oxidase like or germin like (PR-16) proteins. OXO is one of the enzymes that can produce H_2O_2 in plants. It releases CO_2 and H_2O_2 from oxalic acid (OA) that fungi release inside the plant cell. OA not only acidifies the plant tissue but also chelates Ca^{2+} from the cell wall, rendering the stressed tissue susceptible to a battery of fungal degradative enzymes. The enzyme OXO was first isolated and characterized from barley and wheat, known as germin [137, 138]. The OXO leads to production of H_2O_2 a defense signal molecule. On one hand accumulation of H_2O_2 is expected to trigger defense response, on the other hand, it improves the tolerance of the host plant against the fungal toxin [202]. They expressed barley oxalate oxidase gene in oilseed rape enhancing its tolerance to phytotoxic effect of oxalic acid. One of the most important OA-generating necrotrophic pathogens is *Sclerotinia sclerotiorum*. It is widely distributed and pathogenic to more than 400 plant species at various developmental stages.

PR-17

PR-17 proteins have been found as an additional family of PRs in infected tobacco, wheat, and barley and contain sequences resembling the active site of zinc metalloproteinases. The encoded proteins from barley (designed Hv-PRs), HvPR-17a and HvPR-17b belong to the plant pathogenesis-related proteins PR-17 [143]. The family includes also NtPRp27 from tobacco [144] and WCI-5 from wheat [145] responsive to viral and fungal infection, respectively. HvPR-17a was studied in barley upon infestation by the bird cherry-oat aphid (*Rhopalosiphum padi*) in different barley lines, both susceptible and resistant. The different responses in resistant and susceptible lines indicated that the induced HvPR-17a might play a role in resistance against aphid infestation [146].

Other Antifungal Protein

New proteins that have antifungal activity but do not clearly fall into any of the above classes are being discovered at a rapid pace. A putative novel family (PR-18) comprises fungus and SA-inducible carbohydrate oxidases, as exemplified by proteins with hydrogen peroxide-generating and antimicrobial properties from sunflower [147]. Cucumber plants (*Cucumis sativus*) produces a chitinase found in both leaves infected with *Colletotrichum lagenarium* and in uninfected leaves up to five leaves above the infected one [203].

This chitinase has a molecular mass of 28 kDa and is classified as an example of PR-8 proteins. Tobacco plants produce a number PR proteins, among them are chitinases belonging to the PR-11 group [204]. These two chitinase groups are related and have molecular masses of 41 and 43 kDa. Both have endochitinase activity toward *T. viridae* and *Alternaria radicina* and show synergy with a tobacco β-1, 3-glucanase against *F. solani* germlings [205]. Not all families seem to be represented in all plant species and occurrence and properties of different members within a family may differ strongly. The mechanism of action of none of these proteins is known.

APPLICATION OF ANTIFUNGAL PROTEINS

Antifungal Proteins as Therapeutics

Antifungal proteins are new source of clinically useful therapeutics from natural occurring sources like plants, animal, bacteria and fungi. They play an important role in protection against the fungal pathogens like *Candida, Aspergillus* and other pathogenic fungi. One of example is, Histatins are a group of antimicrobial peptides, found in the saliva of man and some higher primates, which possess antifungal properties. Histatins bind to a receptor on the fungal cell membrane and enter the cytoplasm where they target the mitochondrion. Histatin 5 is the most active histatin against the pathogenic yeast *C. albicans,* which is capable of causing lesions in the mouths of immuno-compromised patients [148]. Another important protein is Lactoferrin (formerly known as lactotransferrin) is a glycoprotein, and a member of a transferrin family, these are isolated from colostrums and milk, proteins that capable of binding and transferring Fe3+ ions [149]. Lactoferrin in combination with fluconazole, was shown to reduce the minimum inhibitory concentration (MIC) at which fluconazole killed a number of clinical isolates of *Candida* species, suggesting that lactoferrin may have a potential use in combination therapy against drug-resistant *Candida* infections [151]. A prerequisite for any application of antifungal proteins is the lack of effects on the host cells. A common method to demonstrate safety and selectivity is a hemolytic assay. The absence of cytolytic activity to red blood cells is generally accepted as proof that the protein can be regarded as safe [152]. Many antifungal proteins have been shown to act synergistically with other antifungal as well as antimicrobial proteins. Synergistic effects can alter the activity or even the species specificity of a protein. For example, the antifungal protein Cecropin B alone has no effect on *Escherichia coli*, but in combination with lysozyme it has been shown to efficiently kill the bacterium [150]. Although synergistic effects could be beneficial for many applications, they also may lead to negative effects, since synergistic interaction with human antifungal and antibacterial proteins might alter the antifungal or antibacterial spectrum.

Crop Protection

Antifungal proteins are the powerful tools in field of agriculture and crop protection. Antifungal proteins (AFPs), ubiquitous components in different plant parts have a broad spectrum of biological activity and play a key role in plant defence against pathogenic fungi by preventing or limiting their spread. They can protect plants from devastating damage caused by fungal pathogens and consequently prevent economic losses. Occurrence of AFPs in seeds might be involved in protecting the seeds during resting or storage and germination [153, 154]. However, one of the most promising tools for crop protection is the use of transgenic plants. Expression of osmotin genes in transgenic potato has resulted in increased resistance to *Phytophthora infestans*, a fungus that is known as the late-blight pathogen on potato [155]. Almost all PR families have been expressed in transgenic plants and these plants have become more resistant to diseases [156].

Chitinases and β-1, 3- glucanases are the most attractive antifungal proteins due to their strong *in vitro* activity. Expression of AFP by transgenic plants has been performed successfully involved in wheat, rice plant, and pearl millet. These plants proved to be less susceptible against their potential pathogens. Significant reduction of disease symptoms have been observed for transgenic wheat, rice plants and pear millet infected with *Erysiphe graminis*, *P. recondita*, and *M. grisea*, and *Sclerospora graminicola*, *Puccinia substriata*, respectively. Direct application of AFP on rice and *Pelargonium* plants leaves protected them against *Magnaporthe grisea* and *B. cinerea* after still two or six weeks of infection. Pre-incubation of tomato roots with AFP protected the plants against *F. oxysporum* [157]. Due to the potential of broad-spectrum resistance from use of barley chitinase with antifungal activity, the chitinase gene could be used to enhance fungal resistance in crop plants such as tobacco, rice, clover and tea [158].

Food Preservatives

There is an increasing demand for food preservatives from natural sources to increase their shelf life. So antifungal proteins have been reported to be used as a food preservatives. Amaranthus seed has potent antifungal activity against *Penicillium roqueforti*, a fungus isolated from contaminated bread [159]. This fungus is a major food spoilage fungus and is somewhat resistant to chemical antifungal preservatives. Therefore AFPs source from edible seeds seem to be promising, low-cost food preservatives probably not only in the bakery industry but also in other food processing industries.

CONCLUSION

The discoveries of numerous antifungal proteins from various natural occurring sources, some of which are discussed above provide a number of research and development directions. Antifungal proteins may be powerful tools in human health, Crop protection and food preservation. However, several aspects have to be thoroughly examined prior a possible application. Activity under physiological conditions, resistance, selectivity and synergistic effects are only a few aspects which have to be clarified prior to application of antifungal proteins. So, there is potential requirement of a novel, potent, antifungal molecules which effective against pathogenic fungi.

CONFLICT OF INTEREST

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

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