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Review Article

The potential of plant proteins as antifungal agents for agricultural applications

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A B S T R A C T

Fungal pathogens induce a variety of diseases in both plants and post-harvest food crops, resulting in significant crop losses for the agricultural industry. Although the usage of chemical-based fungicides is the most common way to control these diseases, they damage the environment, have the potential to harm human and animal life, and may lead to resistant fungal strains. Accordingly, there is an urgent need for diverse and effective agricultural fungicides that are environmentally- and eco-friendly. Plants have evolved various mechanisms in their innate immune system to defend against fungal pathogens, including soluble proteins secreted from plants with antifungal activities. These proteins can inhibit fungal growth and infection through a variety of mechanisms while exhibiting diverse functionality in addition to antifungal activity. In this mini review, we summarize and discuss the potential of using plant antifungal proteins for future agricultural applications from the perspective of bioengineering and biotechnology.

1. Introduction

The management of plant diseases is one of the top priorities of the agricultural industry due to the major economic and biosecurity threats that result from plant pathogens [1]. Among the pathogen cortege that crops are afflicted by, fungal infections pose one of the largest risks to food production [2]. Devastating crop failures due to these pathogens, such as the historical and infamous Irish Potato Famine [3] and contemporary issues of rice blast and wheat rust threaten food security and result in major economic losses [4]. The development of fungicides has undoubtedly eased the burden of diminished food security through a reduction in crop failures by successfully controlling fungal diseases. Chemical fungicides, made from either organic or inorganic chemicals, remain as the primary treatment towards most fungal pathogens [5]. However, chemical fungicides have long been documented for their adverse effects on both the environment and animal health [6], and harvested crops must meet strict criteria to ensure chemical residues are found at safe levels for consumptions [7]. Though conventional fungicides have made positive strides in food security and agricultural disease control, the risks they carry need to be addressed and alternative methods of fungal control should be considered.

One common alternative to conventional fungicides is the usage of genetically modified (GM) crops. Transgenic technology has led to the development of crops with desirable traits, such as improved flavor [8], increased yield [9], and superior disease resistance [10] compared to non-modified crops. Notably, the use of transgenic crops permits for a significant reduction in the quantity of phytosanitary product applied to the field [11]. However, the public is often apprehensive about GMO safety and has difficulty accepting genetically modified crops [12]. For example, some consumers believe that GM crops carry more risks than benefits and are willing to pay a premium for foods labeled as non-GMO [13]. Likewise, since 2001, the EU has placed a *de facto* moratorium on approvals of GMOs [14]. Another major concern includes the potential that transgenic crops could damage the ecosystem in unpredictable ways. GMOs can invade ecosystems due to an increase in stress tolerance, causing wild plants to become weeds through horizonal gene transfer [15], or produce toxic substances to pests that may affect nontarget organisms [16]. Recently, increases in pest resistance towards GM crops have also posed problems to the durability of current transgenic crops [17].

Thus, it is necessary to seek alternative antifungal agent candidates that can be applied exogenously as conventional fungicides. These alternative candidates should be environmentally friendly and potentially have fewer negative health impacts on animals than conventional fungicides if applied exogenously. Plants have evolved diverse mechanisms to defend against fungal infections, as summarized in Fig. 1, with one important route utilizing the secretion of proteins to delay fungal infection or inhibit fungal growth. These plant antifungal proteins are

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promising candidates since they are biodegradable, generally nontoxic to humans and antagonistic microorganisms, and most importantly, have evolved for millions of years to combat phytopathogenic fungi with a narrow target range [18]. In this mini review, we summarize and discuss plant defensive proteins that are promising candidates for the development of future antifungal agents for agricultural applications (as summarized in Table 1).

2. Pathogenesis related proteins

Pathogenesis related (PR) proteins are a group of low molecular weight plant proteins involved in mitigating both biotic and abiotic stresses [19], and are often involved in triggering systemic acquired resistance in plants [20]. There are 16 main groups of PRs (PR-1 to PR-16), with each group classified based on different molecular and physiological properties. These proteins are often pathogen specific and involved in the transcriptional activation of plant defenses [21]. Here, we will focus on some of the most promising candidates for the development of antifungal agents for agricultural applications: PR-3, PR-5, PR-6, and PR-12.

2.1. Chitinases (PR-3)

One of the best known and most studied plant antifungal proteins is chitinase, which belongs to PR-3 [22]. Chitinases are strongly induced when the host plants are under attack from pathogens and function as defense molecules against fungal infection [23]. These proteins inhibit fungal growth by lysing hyphal tips in fungi and break down chitin into its oligomers [24]. Chitinases display strong antifungal activity against a wide range of phytopathogenic fungi. This includes *Botrytis cinerea* [25], a necrotrophic fungi that is considered one of the top fungal pathogens based on scientific and economic importance and infects over 200 species worldwide [26], as well as *Rhizoctonia solani* [27], which causes sheath blight in rice, one of the most widespread diseases of rice [28]. While chitinases have been isolated from bacteria [29], fungi [30,31], humans [32], and plants [33], chitin has not been found in mammals and plants [34]. As such, plant chitinases, are a valuable target for developing highly specific biocontrol against phytopathogenic fungi in agriculture.

While overexpressing plant chitinases in either native or heterologous plants have successfully enhanced plant resistance against phytopathogenic fungi [35], plant chitinases have also been used to treat fungal infections as an exogenously applied pest control agent. One study extracted chitinase E from yam tubers and then sprayed it on strawberries infected with powdery mildew. The treatment using chitinase E was successful at preventing the disease for at least two weeks through damaging cell-wall components of the hyphae and conidia of the pathogenic fungi [36]. Plant chitinase has been heterologously expressed in many microorganisms such the bacteria *Escherichia coli* [22] and *Bacillus* sp. [37], the yeast *Pichia pastoris* [38], as well as in plants such as transgenic tobacco [39] and cultured plant cells [40], which provides solid foundation to develop chitinases as antifungal agents. Meanwhile, fermentation optimization has also been explored to enhance the production of chitinase from microbial cell factories, and the strategies include but not limited to adjusting carbon sources, pH, aeration, and temperatures [41].

Importantly, chitinases also display excellent protein stability that ensures reliable exogenous application. Chitinase from *Vitis vinifera* exhibits a half-life of up to 4.7 days at 30 °C or 9 years at 15 °C [42], and the purified chitinase from *Trichosanthes dioica*, effective against *Aspergillus niger* and *Trichoderma* sp. In a fungal agar diffusion assay, remained stable between pH 5.0–11.0 and temperature 30–90 °C for at least 30 min [43]. Likewise, the purified chitinase from *Diospyros kaki*, which inhibited the growth of *T. viride*, exhibits broad pH stability from pH 4.0–9.0, and retains more than 60% activity at pH as low as 3.0 and as high as 10.0 [44]. Due to its specificity in targeting chitin, success in

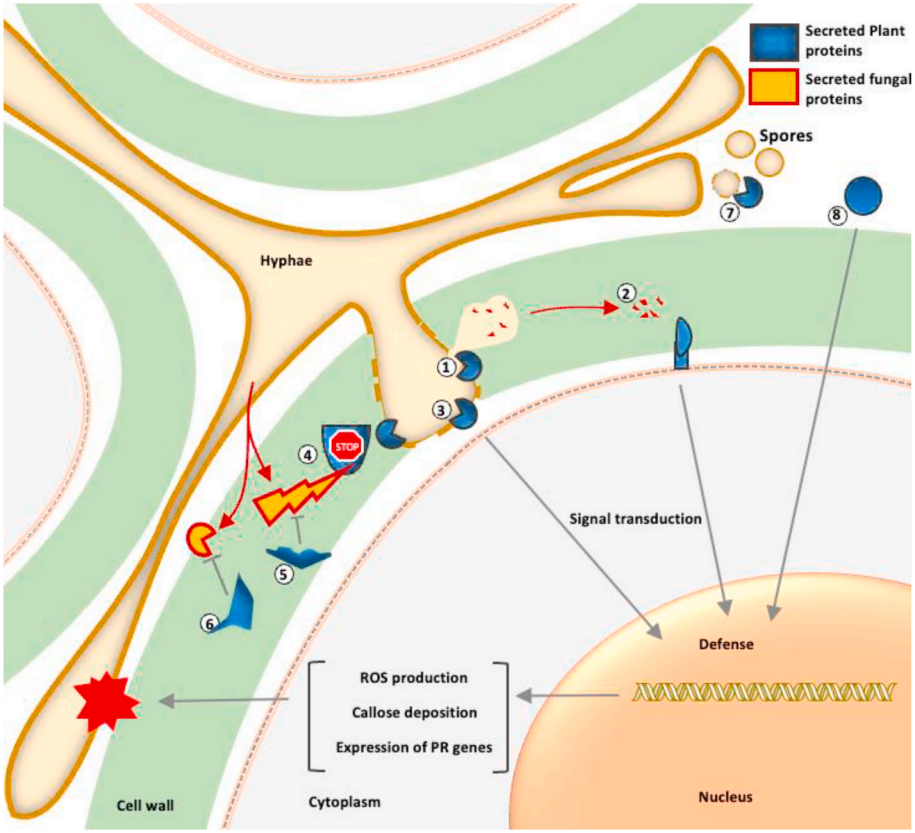

Fig. 1. Mode of actions of secreted plant antifungal proteins with potential agricultural applications. 1) Secreted antifungal proteins reduce fungal hyphae growth by compromising the fungal cell wall and membrane integrity, leading to potential cytoplasmic leakages [165]. 2) Antifungal protein activity generates residues considered as microbe-associated molecular pattern molecules that can be recognized by plant receptors to stimulate plant immune response [166]. 3) Plant antifungal proteins, upon interacting with the target, directly stimulate plant immune response [167]. 4) Plant secreted proteins protect antifungal proteins from cleavage by fungal protease [168]. 5) Inhibition of fungal protease by plant secreted inhibitors [169]. 6) Inhibition of fungal cell wall hydrolase by plant secreted inhibitors [170]. 7) Spore degradation or reduction of germination rate by secreted plant antifungal proteins [171]. 8) Small secreted peptides enhance the efficacy of plant defense [172].

Table 1
Summary of antifungal proteins of potential to be developed into alternative fungi control agents for agricultural applications.

Protein Class	Protein		Exogenous Application Inhibition	Antifungal Mechanism	Ref
Pathogenesis Related Proteins	Chitinase		*Alternaria* sp. *Aspergillus niger* *Botrytis cinerea* *Collectrichum falacatum* *Fusarium* sp. *Pestaloatia theae* *Rhizoctania solani* *Sphaerotehco humuli* *Trichoderma* sp.	• Degradation of chitin via hydrolysis of the N-acetylglucosamine polymer • Lysing of fungal hyphal tips	[25,27,36,43,154–158]
	Defensins	NRBAP	*Mycosphaerella arachidicola*	• Unknown	[50]
		MsDef1	*Fusarium graminearum*	• Ion channel inhibition	[54]
		NaD1	*Fusarium oxysporum f.sp vasinfectum* *Thielaviopsis basicola* *Aspergillus nidulans* *Leptospheria maculan*	• Interacts with fungal cytoplasmic agents • Disrupts plasmic membrane integrity	[56,57]
		RsAFP2	*Fusarium culmorum* *Nectria haematococca* *Verticilium dahlia* *Phoma betae*	• Disrupts plasmic membrane integrity	[58]
	Osmotin and Osmotin-Like Proteins		*Alternaria solani* *Biopolaris maydis* *Bioplaris zeicola* *Cerospora zeae-maydis* *Colletotrichum laginarium* *Colletotrichum sublineolum* *Fusarium graminearum* *Fusarium moniliforme* *Fusarium oxysporum* *Fusarium roseum* *Kabatiella seae* *Phytophthora infestans* *Phytophtora parasitica* *Sclerotinia sclerotiorum* *Stenocarpella maydis* *Trichoderma longibrachiatum* *Verticillium dahliae*	• Inhibition of cell wall barriers via signal transduction pathway • Reduction of pathogen toxicity towards host • Disruption of fungal cell walls, fungal hyphael, and spore germination • Hydrolyse β-1,3-glucans • Fungal membrane permealization	[62,69–71,159]
Protease Inhibitors	Potide-G		*Candida albicans* *Rhizoctania solani*	• Competitive and noncompetitive inhibition of serine, aspartic, and cysteine proteases	[76]
	Potato Protease Inhibitor I and II		*Botrytis cinerea* *Fusarium oxysporum* *Fusarium solani*	• Chymotrypsin and serine protease inhibition	[75,78,160]
	Bowman-Birk Protease Inhibitor		*Fusarium culmorum* *Fusarium graminearum* *Mycosphaerella arachidicola* *Septoria tritici*	• Protease serine protease inhibitor • Noncompetitive inhibition of trypsin and chymotrypsin	[92,93,161,162]
Antimicrobial Peptides	Prosystemin		*Botrytis cinerea*	• Induces protease inhibitors • Amplifies defense signaling process for wounded plants	[104,105]
	StSN1		*Botrytis cinerea* *Fusarium* sp.	• Mechanism unknown	[111,163]
	Puroindoline A and B		*Alternaria brassicola* *Ascochyta pisi* *Fusarium culmorum* *Fusarium graminearum* *Magnaporthe girsea* *Rhizoctania solani* *Verticillium dahlia*	• Induces membrane instability	[116,164]
DUF26-Containing Proteins	Ginkbilobin2		*Candida albicans* *Fusarium oxysporum* *Trhicoderma reesei*	• Binds sugar motifs on hyphal surface	[122,123]
	AFP1/AFP2		*Ustilago maydis*	• Binds to sugar motifs on hyphal surface	[124]
	VdCRR1		*None tested*	• Stabilize/protect chitinases from fungal proteases	[125]
	TaCRR		*Bipolaris sorokiniana Rhizoctania cerealis*	• Inhibited mycelia growth, activation of pathogenesis-related genes	[128]
Leucine Rich Repeat Protein	PvPGIP2		*Aspergillus niger* *Botrytis cinerea*	• Competitive and noncompetitive inhibition	[144]
S Albumin	2S Albumin and 2S Albumin Orthologs		*Aspergillus flavus* *Aspergillus fumigatus* *Candida albicans* *Fusarium oxysporum* *Phanerochaete chrysosporium* *Trichoderma harizanum*	• Mechanism unknown	[146,150–152]

both plant extraction and heterologous expression from microbial factors, and numerous studies documenting its antifungal efficacy, chitinase is a promising candidate to develop as an antifungal agent. However, compared to the number of available chitinase studies that use transgenic plants, the exogenous application of chitinases on infected plants remain to be more thoroughly investigated.

2.2. Defensins (PR-12)

Defensins belong to group PR-12 [45], and exhibit broad-spectrum activities against different biotic agents including pathogenic fungi [46]. They are named due to the structural and functional similarities to insect and mammalian defensins [47]. Plant defensins are constitutively expressed in the extracellular space of most vegetative and reproductive plant tissues [48] and can be specifically induced under pathogen stress condition [47,49]. Typically, defensins are small soluble cationic proteins, 45–54 amino acid residues in size, exhibiting eight conserved cysteine residues (C1 to C8) with a conserved spacing pattern, and the tertiary structure is supported by at least four disulfide bonds [46]. Defensins remain stable both under extreme temperatures (as high as 90 °C) and very acidic conditions (pH as low as 1) [48]. For instance, NRBAP, a defensin-like protein purified from *Phaseolus vulgaris* beans, retained its antifungal activity against *Mycosphaerella arachidicola* up to 100 °C, and in the pH range of 1–13 [50].

Defensins can interact with a significant diversity of biological targets (e.g., proteases [51], protein synthetic machinery [52], α-amylases in insects [53], and ion channels in fungi [54]). One common mechanism that defensins often adopt to inhibit fungal growth is through the disruption of cell plasma membranes. Plant defensins are usually positively charged proteins and interact with anionic moieties in the membrane, such as glycoproteins, sphingolipids, or phospholipids [49]. The defensins cover the target membranes until it reaches a concentration threshold, and then disrupts the membrane integrity by affecting the bilayer curvature [55]. One study provided evidence that NaD1 from *Nicotiana alata*, which displays antifungal activity against several agronomically important filamentous fungi [56], was able to bound to phospholipids phosphatidic acid [57]. To estimate the effect of the total net charge of defensins on the antifungal activity, a mutagenesis analysis was performed on Rs-AFP2 from radish, and the interaction between the defensins and membrane lipids was improved when the net charge of the protein increased [58]. Plant defensin antifungal activity may not be restricted to targeting the membrane of pathogenic fungi. Indeed, the exogenous application of NaD1 is also associated with the entrance of the protein into fungal intracellular space, resulting in granulation of the cytoplasm and cell death [56]. This suggests that plant defensins could also interact with fungal intracellular targets and possibly with DNA, as already demonstrated by ostrich β-defensins, where *E. coli* growth was inhibited in assays due to interactions between peptides and cytoplasmic targets that curbed DNA, RNA, and protein synthesis [59]. The diversity of antifungal mechanisms and effectiveness of defensins against a wide range of pathogens implies the potential of this protein family as a promising resource for fighting plant pathogens.

2.3. Thaumatin-like proteins (PR-5)

Thaumatin-like proteins (TLP) belong to PR-5 family [60]. TLPs are named so due to their structural similarity to thaumatin, a sweet-tasting, non-toxic protein that was first discovered from the fruit of the tropical plant *Thaumatococcus daniellii* [61]. TLPs exhibit a broad range of biological activities, including antifungal activity. Different TLPs inhibit fungal growth through different mechanisms, including but not limited to disrupting fungal membrane [62], inhibiting fungal enzymes such as xylanase [63], inducing apoptosis by binding to specific fungal membrane receptors [64], and hydrolyzing β-1,3-glucans [65]. Osmotin and osmotin-like proteins are among the most studied TLPs of antifungal activity [66]. Osmotin and orthologs have been shown to exhibit broad-spectrum antifungal inhibitory effects [67]. Overexpression of osmotin in transgenic plants delayed disease symptoms from fungal pathogens [68]. Osmotin isolated from tobacco cell suspensions can inhibit the hyphal growth of numerous pathogenic fungi *in vitro*, including species from *Bipolaris*, *Collectorichum, Fusarium*, *Kabatiella, Phytophthora*, and *Trichoderma* [69]; and another osmotin-like protein from *Solanum nigerum* and overexpressed in *E. coli* can inhibit the growth of phytopathogenic *Fusarium solani f. sp. glycines*, *Macrophomina phaseolina*, and *Collectrichum glaesporioides*, and *Collectrichum gossypii* var. *cephalosporioides* at the concentration between 0.1 μg/μL to 0.3 μg/μL [70]. Additionally, an osmotin-like protein from *Solanum nigrum* L. var *indica* was shown to inhibit fungal spore germination and permeabilize fungal hyphae *in vitro*. This protein is also stable and retains its antifungal activity at temperatures as high as 75 °C for 30 min and pH 3–8 [71]. Further functional exploration of TLPs under various stress conditions for *in planta* assays will be necessary before its development into a reliable antifungal tool [72].

2.4. Protease inhibitors (PR-6)

Plant protease inhibitors (PIs), also called PR-6, are important proteins involved in many plant biological processes, including seed germination, protease-related house-keeping functions, and defense against biotic and abiotic stresses [73]. PIs are normally found in ample quantities in seeds and tubers, and plants in the *Solanaceae* family generally have exceptionally high levels of PIs [74], including some that can be promising candidates of antifungal agents. For instance, potatoes encode several PIs ranging from 4.1 to 39 kDa that exhibit broad-spectrum antifungal activities [75]. Potide-G, a Kunitz-type PI isolated from potato tubers of size 5.5 kDA, inhibits pathogenic fungi *Candida ablicans* and *Rhizoctania solani in vitro* even when heated to 70 °C for 20 min, and also exhibits antiviral and antibacterial activities [76]. Similarly, the potato protease inhibitors I and II (PPI–I and PPI-II) can inhibit the growth of various fungi, including *B. cinerea* [77], *Fusarium solani*, and *Fusarium oxysporum* [75]. Both PPI-I and II are heat stable, which can maintain their ability to inhibit *F. solani* and *F. oxysporum* growth *in vitro* under temperature as high as 100 °C [78]. PPI-I and II are also nontoxic, as they have previously been utilized in human clinical trials for appetite control [79]. The extraction of bioactive PPIs from potatoes is laborious and of low yields [80]. They have also been heterologously expressed in *Saccharomyces cerevisiae,* yet the antifungal activity of the purified protein was not examined [81]. A more economic production method is needed to enable the development of PPIs as antifungal agents for agriculture applications.

Another PI of interest is the Bowman-Birk protease inhibitor (BBI), which is typically under 20 kDA [82,83], contains seven conserved disulfide bonds, and inhibits trypsin and chymotrypsin, which are common enzymes pathogenic fungi utilize when infecting plants [84]. The BBI gene is induced during plant immune responses and overexpression of this gene in plants confers improved disease resistance against both insect and fungal pathogens [85]. BBIs from the legume (*Fabaceae*) or cereal (*Poaceae*) family have a double or single inhibitory loop respectively [86], and synthetic peptides that contain only the disulfide-linked, 9-residue long loop have shown to retain their trypsin and chymotrypsin inhibitory activity [87]. This short, truncated form of the protein may be of interest for the development of antifungal agents of smaller molecular weight for easier production and higher stability, compared with larger protein agents. Aside from the small size, BBI is thermostable with the ability to withstand 100 °C for 10 min, tolerates a wide pH range from 1.6 to 8.0, is not allergenic, and is approved by the FDA for human consumption [88]. Additionally, unlike some other candidates to be engineered as antifungal agent, BBI has passed phase II human clinical trials and is highly unlikely to be toxic, especially given its prevalence in soy products [89]. BBIs have already been successfully utilized as an exogenously applied antifungal agent *in vitro*. One study identified that a BBI-type trypsin-chymotrypsin inhibitor purified from broad beans can

inhibit the growth of *B. cinerea, F. oxysporum,* and *M. arachidicola* at a dose as low as 60 μg per plate [90]. Plant BBIs have often been isolated from a variety of seeds such as those from *Vigna mungo* [91], *Cajanus cajan* [92], *and Clitoria fairchildiana* [93] and have been tested for their insecticidal properties. Rice BBI has also been expressed in *E. coli* and retained the inhibitory activity. However, the titer is relatively low at 20 mg/L, likely due to the presence of the disulfide bonds that make it prone to forming inclusion bodies [94]. In addition, care should be taken when developing BBI as an antifungal agent, as it is a multifunctional PI with a relatively broad activity towards various proteases [95], and may affect beneficial microbiota and fungi in the soil and plants.

3. Antimicrobial proteins

In addition to PRs, antimicrobial peptides (AMPs) are another protein group of interest. AMPs, also known as host defense peptides [96], can be derived from a variety of organisms, including plants, bacteria, and fungi. In plants, AMPs play a role in the plant innate immune system [97]. AMPs that work specifically against fungi are known as antifungal peptides (AFPs) [98], and feature a wide range of functions that are of interest to both pharmaceutical [99] and agricultural industries [100]. Here, we will only discuss AFPs that have shown potential for agricultural applications.

One AFP of interest is tomato systemin, a small peptide of only 18 amino acids long and is involved in inducing the synthesis of PIs in response to plant wounding and damage from herbivores [101]. Research suggests that systemin moves through the plant phloem and helps amplify the signaling process and allows for distal leaves to respond to the wounding [102]. Tomato plants that overexpress prosystemin, the precursor of systemin, are found to induce high levels of PI proteins even without wounding [103]. Additionally, transgenic plants expressing prosystemin reduce lesions by at least 50% from *Phytophthora infestans,* a pathogen that causes late blight [104]. Systemin peptides have been successfully isolated from tomato, sprayed onto grapevine (*Solanum melongena*) and eggplant (*Vitis vinifera*) plants infected with *B. cinerea* [105] at a concentration of 100 pM, and efficiently delayed necrosis of the infected plants.

Snakins are cell wall-associated defensins that are also classified as AMP and believed to play a role in plant growth, signaling, and defense [106]. Snakins isolated from *Solanum tuberosum* (StSN1) are cysteine-rich peptides roughly 6.9 kDA in size [107,108] and the snakins isolated from potato tubers is effective at suppressing both fungal and bacterial growth at concentrations lower than 10 μM [109]. Transgenic potato plants overexpressing the *StSN1* gene exhibited reduced symptoms of *R. solani* infections and higher survival rates compared to the wild type plants [110]. Additionally, StSN1 has been shown to be effective *in vitro* against *B. cinerea* and several *Fusarium* species [111] However, snakins have been rarely expressed successfully from microbial hosts, often with low yield and insolubility, which hinders in-depth mechanistic characterization of its action towards pathogenic fungi [112].

Another promising group of AFPs are the puroindolines, which are small, amphipathic tryptophan-rich proteins about 13 kDA in size and found only in wheat (*Triticum*) [113]. They are known to inhibit the growth of pathogenic bacteria and fungi with low mammalian toxicity [114], likely through strong binding with microbial membranes and therefore perturbing the membrane integrity [115]. The primary roles of puroindolines include grain hardness and fungal defense. These proteins are believed to protect seeds from fungal attacks during seed development and germination [116]. There are two major puroindolines, Puroindoline A (PINA) and B (PINB) [117]. When the *pin* genes are overexpressed in transgenic rice, rice displayed significantly enhanced resistance to rice blast caused by *Magnaporthe grisea* and a reduction in symptoms due to *Rhizoctonia solani* infections [118]. Purified PINA and PINB proteins from wheat were able to inhibit the growth of a variety of pathogenic fungi, including *Alternaria brassicola, Ascochyta pisi, F. culmorum, F. graminearum, Magnaporthe girsea, R. solani,* and *Verticillium dahlia*. PINA and PINB are stable over a broad range of temperature (70 °C–130 °C) and pH (2.0–12.0) [115,119]. PINs have been heterologously produced in *Pichia pastoris* with a titer up to 14 mg/L taking advantage of puroindoline's solubility in the detergent Triton X-114 [120]. These various AFPs discussed highlight the potential of using AFPs as antifungal agents for agricultural purposes.

4. DUF26-containing proteins

In the past decade, there has been an increase in interest towards proteins containing domain of unknown function (DUF26) for their capability in fighting plant pathogens and especially fungi [121]. DUF26 is a cysteine rich domain with a conserved C-X8-C-X2-C motif. DUF26-containing proteins are a large, land plant-specific protein family and characteristic of embryophytes [121]. Similarities with fungal lectins suggests DUF26-containing proteins constitute a group of plant carbohydrate-binding proteins able to recognize specific fungal sugar motifs [121].

There are three groups of DUF26-containing proteins: the cysteine-rich receptor-like secreted proteins (CRRSPs), cysteine-rich receptor-like kinase (CRKs) and plasmodesmata-localized proteins (PDLPs). The three DUF26-containing protein groups were all previously associated with antifungal activities. Nevertheless, only CRRSPs remain as good candidates for biotechnological application since CRKs and PDLPs contain transmembrane domains and localize to the membranes. CRRSPs contain a signal peptide followed by one or more DUF26 domains, separated by a variable region [121]. The most well-known CRRSP is Ginkbilobin2 (Gnk2), which was isolated from seeds of *Ginkgo biloba* and able to inhibit the growth of *F. oxysporum*, *T. reesei*, and *C. albicans* [122]. This antifungal activity is likely due to the binding of DUF26 domain with sugar moieties on the fungal cell wall [123]. For instance, Gnk2 interacts specifically with mannan, a yeast cell wall polysaccharide, and mannose, a building block of mannan, by strictly recognizing the hydroxy group at the C4 position of the monosaccharide. Consistently, two maize CRRSPs (AFP1 and AFP2) have been characterized to interact directly with the hyphal surface of *Ustilago maydis*, and the activity can be rendered by Rsp3, a *U. maydis* effector covering its surface [124].

In addition to direct binding with fungal cell walls, DUF26-containing proteins from CRRSP family also protect plants using indirect mechanisms. CRR1, a secreted apoplastic protein from cotton, and composed of two Cys-rich DUF26 motifs, interacts and protects the antifungal apoplastic chitinase 28 from cleavage by VdSSEP1, a pathogen related protease [125]. Importantly, overexpressing CRR1 in heterologous plants such as *Arabidopsis thaliana* and *Nicothiana tabacum* improved plant resistance to *B. cinerea* and *P. parasitica,* respectively. Thus, CRR1 could be a good candidate as a co-antifungal agent and simultaneous exogenous application of CRR1 and chitinases should be evaluated. Another CRRSP of interest is the recently reported CBM1-interacting protein (OsCBMIP) in rice [126]. Pathogenetic fungi generally use cell wall degrading enzymes (CWDEs) to destruct plant cell walls, and many CWDEs use carbohydrate binding modules (CBMs) to facilitate the access to plant polysaccharides to advance the infection process [127]. OsCBMIP can specifically bind to CBM of several CBM-containing CWDEs including the xylanase MoCel10A of the blast fungus pathogen *Magnaporthe oryzae* and slow down the infection progress. Interestingly, OsCBMIP cannot inhibit the growth of *M. oryzae* and *F. oxysporum in vitro*, and this further indicates that OsCBMIP slows down the infection of pathogenetic fungi through indirect mechanism, here specially, through inhibiting CBM-containing CWDEs [126]. In another study, a transcriptomic analysis of wheat after *Bipolaris sorokiniana* or *Rhizoctania cerealis* infection reported the induction of a cysteine-rich protein (CRR), TaCRR [128]. When heterologously expressed, this DUF26-containing protein showed a clear antifungal activity. Besides, it was found that silencing *TaCRR* gene in wheat

significantly decreased the expression of pathogenesis-related genes such as *β-1,3-glucanase*, *defensin* or *chitinases* [128]. Owing to their apoplastic localization and direct or indirect antifungal activities, DUF26-containing proteins from the CRRSP class remain as attractive candidates for the future development of antifungal agents.

5. Other proteins

Polygalacturonase inhibiting proteins (PGIPs) are a family of leucine rich repeat (LRR) proteins found in plant cell walls [129,130] whose primary role is to inhibit polygalacturonases (PGs), enzymes secreted by insects and fungal pathogens that degrade the plant cell walls and leave it vulnerable for infection [131]. Through competitive or noncompetitive inhibition, PGIPs slow the hydrolysis process of PGs [132–135]. Presently, numerous studies show that overexpression of PGIPs in transgenic plants leads to increased fungal resistance. The best-documented PGIP is PGIP2 from *Phaseolus vulgaris* (PvPGIP2), the common bean [136]. PvPGIP2 has been successfully expressed in transgenic plants, resulting in increased resistance to fungal infections against *Alternaria citri, Aspergillus flavus*, *A. niger*, *B. cinerea*, *Claviceps purpurea*, and *F. graminearum* [137–140]. Similarly, expression of PGIP3 from soybeans (*Glycine max*) in tobacco has been shown to inhibit the growth of pathogenic *Sclerotinia sclerotiorum, Fusarium moniliforme, B. aclada, A. niger, Collectotrichum acutatum,* and *F. graminearum* [141,142]; and expressing PGIP2 from lima beans (*Phaseolus lunatus*) in tobacco also delayed growth of *Collectrichum lupini*, *B. cinerea*, *F. moniliforme*, and *A. niger* [143]. Recently, it is also found that truncated PvPGIP2 with only the optimal docking area retains similar level of inhibitory activities towards PGs from *A. niger* and *B. cinerea* to the full-length PvPGIP2 [144]. Yeast strains secreting full-length or truncated PvPGIP2 with the *Ost 1* signal peptide were also able to reduce fungal growth and delay sporulation by 1–2 days [144]. Although the function of PGIPs when applied exogenously on plants has not been reported, this group of proteins is still considered as a promising candidate to be developed into an eco-friendly fungal control agent.

Albumins are a major class of water soluble, seed storage proteins that are used as a source of nutrients for plants during germination [145]. Among them, 2S albumins have antifungal capabilities [146], in addition to a variety of activities including anti-cancer, anti-fungal, anti-bacterial, and serine-protease inhibiting properties [147]. These small storage proteins are present in both monocotyledonous and dicotyledonous plant seeds [148] and typically have a disulfide bridge linking two different subunits, which are typically between 3 kDA and 10 kDA in size [149]. For example, pumpkin (*Cucurbita* sp.) 2S albumin is thermal-stable at up to 90 °C, and exhibits inhibitory activity against the fungal pathogen *F. oxysporum* [147]. Similarly, a crude extract of peanut (*Arachis hypogaea*) containing 2S albumin was found to inhibit growth of *A. flavus* [150]; the 2S albumin ortholog from passionfruit (*Passiflora edulis*) could also inhibit the fungal pathogens *T. harizanum* and *F. oxysporum* [151], *C. musae*, and *C. lindemuthianum* [146]; and the 2S albumin ortholog from *Putranjiva roxburghii* (putrin) could inhibit the growth of *F. oxysporum*, *Phanerochaete chrysosporium*, *C. albicans*, *Aspergillus fumigatus*, and *A. flavus*. In addition, putrin is stable at up to 50 °C and within a pH range from 6 – 8 [152]. On the other hand, 2S albumin from white sesame seeds, oriental mustard, and Brazil nuts can bind to IgE sera, which may trigger an allergic response in humans [145]. Thus, before 2S albumin can be utilized as an exogenously applied antifungal agent, we need to either engineer the protein to eliminate or reduce the allergenicity or modify the application in a manner that avoids either extensive contact or consumption.

6. Conclusion

Chemical-based fungicides are known to be detrimental to the environment and may lead to resistance in pathogenic fungi [153]. Unlike chemical fungicides, the use of exogenously applied natural plant proteins with known antifungal properties can potentially be an eco-friendly and sustainable method for controlling fungal diseases. These natural plant proteins are more socially acceptable, and compared with the production of transgenic plants, are more flexible. Additionally, antifungal plant proteins offer a variety of mechanisms and tools, urgently needed to fight against the rapidly evolving fungal pathogens. As summarized in Table 1, these naturally occurring plant peptides are strong candidates for developing broad-spectrum, fungal-control strategies. One of the biggest hurdles to consider when developing these proteins is lowering the cost of production while enabling mass production. It will be necessary to explore and further optimize microbial factories and protein extraction methods before many of these natural plant proteins can be utilized readily in agricultural industry. Numerous studies showcase the efficacy of these proteins both *in vitro* and *in planta* against pathogenic fungi. The potential of using natural plant proteins exogenously to control agricultural fungal diseases remains largely untapped and need to be considered when developing future eco- and environmentally-friendly antifungal agents.

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