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Harnessing antifungal proteins from fungi to protect plants: a review of the *Epichloë festucae* antifungal protein *Efe*-AfpA

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Abstract

The *Epichloë* fungal endophytes of turfgrasses have long been studied for the benefits they provide their plant hosts such as enhancing the host's physiological characteristics and providing disease resistance against fungal pathogens. The mechanisms of fungal disease resistance have been attributed to outcompeting of nutrients or through antifungal secondary metabolites, but these mechanisms have not been validated. The well-established endophyte mediated disease resistance in strong creeping red fescue (*Festuca rubra* subsp. *rubra*) against dollar spot disease caused by *Clarireedia jacksonii* is due to the presence of the endophyte *Epichloë festucae*. Studying this tripartite relationship has led to the identification of an antifungal protein designated *Efe*-AfpA. Expression, purification, and testing of this protein on *C. jacksonii* in culture and on infected turfgrasses has verified *Efe*-AfpA's role in the endophyte-mediated disease resistance. Several other antifungal proteins similar to *Efe*-AfpA have been characterized from other fungal species and could represent an untapped, novel control method for fungal plant diseases.

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Introduction

Microbes and plants are known to form close associations and symbioses with each other. These associations can either be mutualistic or antagonistic depending on the pairing. Many grasses from the family Poaceae will form these relationships with Clavicipitaceous fungi, particularly species from the genus *Epichloë*[1]. A common benefit of these fungal endophytes is the reduction in herbivory by animals due to the alkaloids produced by the *Epichloë* species[2]. *Epichloë* species have also been shown to have antifungal activity against other fungi, mostly through *in vitro* dual growth cultures[3].

A unique benefit of the *E. festucae* – *Festuca rubra* subsp. *rubra* symbiosis is the endophyte-mediated disease resistance the grass host attains against dollar spot disease, caused by the fungal pathogen *Clarireedia jacksonii*, and red thread, caused by *Laetisaria fuciformis*[4,5]. Dollar spot disease is a highly detrimental foliar disease of turfgrasses requiring the use of both chemical and cultural control methods[6]. The use of chemical controls often leads to the selection of resistant strains, so the discovery of novel control methods that could be utilized in tandem with, or in replacement of fungicides, would be a boon to growers. As such, understanding the underlying mechanism of the endophyte-mediate disease resistance imparted by *E. festucae* represented an opportunity to discover such a novel control method. Using transcriptome analysis and protein expression systems, an *E. festucae* antifungal protein, *Efe*-AfpA, was identified and its activity against *C. jacksonii* was verified[7–9].

The purpose of this review is to summarize the identification of the antifungal protein *Efe*-AfpA and discuss the attempts of using it and other antifungal proteins to suppress plant pathogenic fungal diseases. To do so, a variety of topics need to be discussed: (1) the *Epichloë* spp. endophytes of grasses, (2) the tripartite relationship between *E. festucae*, *F. rubra* subsp. *rubra*, and *C. jacksonii*, (3) *Efe*-AfpA and other antifungal proteins, and (4) how these antifungal proteins could be a new source of control for fungal plant diseases.

Epichloë endophytes and their benefits

Endophytic relationships between fungi and plants have been well documented for years. For the most part, these fungi have been described to be from two distinct groups, Clavicipitaceous and Nonclavicipitaceous that are further divided into classes[10]. Relationships between these fungi and grasses can range from antagonistic to symbiotic, such as the case of the *Epichloë* species of the *Clavicipitaceae* fungi. These fungi endophytically interact with cool season grasses of the genera *Agrostis*, *Brachypodium, Bromus*, *Elymus*, *Festuca*, *Lolium*, and *Poa*[11]. There is considerable host specificity among the *Epichloë* spp., which is reviewed in Leuchtmann et al.[12].

Endophytic production of bioactive molecules provides protection to the hosts from a variety of stresses. Peramines, indole diterpenes, ergot alkaloids, and lolines have all been identified as being synthesized by the *Epichloë* endophytes[13–16]. These compounds protect the plant against animal and insect feeding. This reduced insect feeding can have the beneficial side effect of the reduction of plant diseases vectored by those insects. Endophyte infected meadow ryegrass (*Lolium pratense*) had a lower incidence of aphids and barley yellow dwarf virus compared to non-endophyte infected grass, which was attributed to the secondary metabolites the endophyte produced[17]. In some cases, endophyte infection also provides tolerance to abiotic stressors through increased drought tolerance, increased root growth and root size[11].

Class I Clavicipitaceous endophytes, which includes *Epichloë festucae*, was the first class shown to contribute fungal disease resistance to their hosts. Specifically, *E. festucae* infecting *Festuca rubra* is one of the few associations that shows strongly associated *in symbio* fungal disease resistance, against dollar spot disease caused by *Clarireedia jacksonii*, and red thread caused by *Laetisaria fuciformis*[4,5,10]. There have been several studies attempting to identify the antifungal capabilities and compounds of these endophytes, which will be discussed later. While there are many reports of *Epichloë*-dependent antifungal activity in dual cultures or using extracts, there are few reports of this activity in planta. Of those reports, most use detached leaf assays or whole plant assays in controlled settings[3]. The *E. festucae* suppression of dollar spot in fine fescues is unique in that it has been observed consistently in the field[4], further solidifying interest in this interaction.

Epichloë festucae – Festuca rubra subsp. *rubra – Clarireedia jacksonii* interaction

In 1989 at the Rutgers Plant Science Research Farm in Adelphia, NJ, USA, strong creeping red fescue infected with the endophyte *E. festucae* was seen to have resistance to dollar spot disease caused by *Clarireedia jacksonii* (formerly *Sclerotinia homeocarpa*)[4,18]. Strong creeping red fescue plants not infected with *E. festucae* did not exhibit resistance.

Dollar spot is a destructive disease of turfgrasses whose diagnostic symptoms and signs include bleached white foliar lesions, sunken depressed circles of turf, and cobweb-like mycelium evident early in the morning on dew covered grass[6]. In the United States, more money is spent on controlling dollar spot disease with chemical fungicides than any other turfgrass disease[19,20]. Specifically, over 70% of all fungicides used for controlling turfgrass diseases are targeting dollar spot, brown patch (*Rhizoctonia solani*), and anthracnose (*Colletotrichum* sp.)[21]. Several chemical fungicides have been used over the years in the control of this disease such as: benzimidazoles, anilazine, dicarboximides, and DMIs (demethylation inhibitors). Unfortunately, resistance has been found for all of these[22]. Fungicide programs are developed to deter the onset of these resistance strains. Application of tank mixes of fungicides (azoxystrobin, chlorothalonil, propiconazole, etc.) with or without the addition of plant growth regulators (paclobutrazol or trinexapaethyl) have shown reduced disease severity, with those including plant growth regulators yielding a higher quality of *Agrostis stolonifera* (creeping bentgrass)[23].

Given that *E. festucae* infecting fine fescues had such a strong association with fungal disease resistance, and it is uniquely conferring disease resistance *in symbio*, the exact mechanism of how this resistance is achieved was investigated. Understanding this mechanism could represent another tool in mitigating the damage of dollar spot disease, both physically and financially. First, a comparative transcriptomic analysis was done on endophyte infected and endophyte free strong creeping red fescue *via* SOLiD-SAGE[7]. The resulting sequence data showed that the fungal endophyte genes for secreted proteins were abundant, and the second most abundant fungal transcript (6.3% of mapped fungal tags) was for a predicted secreted, small, cysteine rich protein with similarity to the *Penicillium chrysogenum* antifungal protein PAF[7]. The *E. festucae* antifungal protein was designated *Efe*-AfpA and the gene was designated *Efe-afpA*, using the *Epichloë* nomenclature guidelines[24].

Of the ten available *Epichloë* genomes at the time, only one other, *E. inebrians*, had this gene present[7]. Since then, genome sequences of additional *Epichloë* spp. became available, which also have an antifungal protein gene, *E. baconii* and *E. aotearoae*[8]. *E. coenophiala*, a fungal endophyte of tall fescue turfgrass also has antifungal protein genes[9]. *E. coenophiala* is a triparental hybrid fungus with two sequences similar to *E. festucae*'s *Efe-afpA* gene. The first is 100% identical, so it can be assumed this gene comes directly from the *E. festucae* parent of *E. coenophiala*. The other sequence is highly similar to *Efe*-AfpA, so it could also be from *E. festucae* or possibly the *Lolium*-associated endophyte component of the triparental hybrid. The third progenitor of *E. coenophiala*, *E. typhina*, does not have an *afpA* gene[7,25,26]. In a search of the NCBI database, only five of the 16 *Epichloë* spp. for which whole genome sequences are currently available have a gene similar to Efe-AfpA. Although *E. coenophiala* has genes similar to *Efe*-AfpA, tall fescue has not been reported to exhibit endophyte-mediated disease resistance likely due to the low expression of the antifungal protein genes[9,27]. There is no information available on disease resistance or gene expression for the other *Epichloë* spp. that have an antifungal protein gene. The limited existence of an antifungal protein gene among *Epichloë* species and the unique nature of the endophyte-mediated disease resistance in *F. rubra* suggested the antifungal protein may be a factor in the disease resistance.

Antifungal proteins

Antimicrobial peptides are common among all organisms ranging from single celled to multicellular organisms, and share a variety of characteristics: small, cationic, and amphiphilic[28]. Of the several known fungal antifungal proteins, AFP and PAF, produced by *Aspergillus giganteus* and *Penicillium chrysogenum* respectively, have been studied most thoroughly.

AFP is a 51 amino acid protein with four disulfide bonds whose NMR structure was determined in 1995. The data showed five beta barrel structures stabilized by four disulfide bonds, but also a level of cysteine pair isomerization[29]. PAF, the other highly researched antifungal protein, is produced by *P. chrysogenum*. It has about 43.6% amino acid sequence identity and 71.3% sequence similarity to AFP and shares the characteristics of being small and cationic, but has three disulfide bonds[30–32]. Both were shown to have high activity against a variety of fungi at low MIC (minimal inhibitory concentrations), another characteristic of these types of antifungal proteins[31,33]. Recently many more of these proteins have been isolated and characterized[8,30,34–43].

PAF, as with many antifungal proteins, has a broad range of antifungal activity from the fungi it affects and the effective concentrations[44]. Some fungi are extremely sensitive to PAF, and to the other *P. chrysogenum* antifungal protein PAFB, such as *Aspergillus fumigatus* and *A. niger*, having MICs of 1 μM or less. There are more resistant fungi, as is the case with *P. chrysogenum* which is extremely sensitive to PAFB but not to PAF[43]. Sensitive fungi have been shown to internalize PAF, where it localizes to the cytoplasm as seen by using immunofluorescence in several *Aspergillus* species. Internalization of PAF has multiple effects on the target fungus, such as membrane permeabilization and changes in morphology such as hyperbranching[31,45]. More recently it was shown that the membrane

sphingolipid glucosylceramide was required for PAF to have full antifungal activity on *Neurospora crassa*, while PAFB did not have the same requirement[46].

AFP from *A. giganteus* is also active against a wide variety of ascomycete fungi such as multiple *Fusarium* and *Aspergillus* species and *Botrytis cinerea*[29,47,48]. This antifungal protein's mode of action has been explored using the Sytox green viability stain, where sensitive fungi treated with the protein would take up the stain. Using both immunofluorescence and an AFP-antibody, it was shown that the protein interacted with the plasma membrane leading to the conclusion that it must permeabilize the membrane allowing for the dye to enter the cells[48]. Transmission electron microscopy of AFP treated *A. niger* (sensitive) and *P. chrysogenum* (resistant) showed major differences in the cellular ultrastructure. Treated *A. niger* showed a collapsing cytoplasm and aberrant vacuoles, with AFP localizing to the cell wall visualized through immunogold staining. *P. chrysogenum* did not show AFP localization to any particular cellular compartment but was internalized, and the ultrastructure remained similar to the untreated control[49].

Analysis of antimicrobial peptides revealed a conserved structure termed the γ-core motif. Variations of this motif are found in antimicrobial proteins, defense polypeptides, and toxins suggesting that the γ-core is an essential component of the membrane interactions of these compounds[50]. Modern molecular and computational modelling work suggests that the AFP protein's γ-core motif is responsible for its interaction with fungal membranes. Furthermore, the modeling showed a likely scenario where AFP proteins would assemble as a blanket over the fungal membrane[51,52]. Similarly to how PAF requires the lipid glucosylceramide for full activity, AFP depends on the C-3 unsaturation of membrane glycosylceramides. Those fungi with Δ3 unsaturation are sensitive to AFP while fungi with Δ3 saturation are insensitive. When the gene responsible for Δ3 unsaturation, *dtdA*, was knocked out in *A. niger* and *Fusarium graminearum* their sensitivity to AFP decreased[53].

Efe-AfpA, *Epichloë festucae* Antifungal Protein A

Epichloë festucae's *Efe*-AfpA was first identified through SOLiD-SAGE analysis of RNA from *E. festucae* infected strong creeping red fescue as the second most abundant fungal tag. It was predicted to be secreted and antifungal by sequence analysis[7]. Protein sequencing of *Efe*-AfpA isolated from the apoplast confirmed its abundance in infected plants. Its predicted amino acid sequence showed high sequence similarity to the well characterized PAF antifungal protein from *P. chrysogenum*. This apoplastic isolated *Efe*-AfpA was tested against *C. jacksonii in vitro* to determine activity and resulted in a zone of inhibition of growth[8].

While other antifungal proteins can be isolated from fermented pure cultures, *Efe*-AfpA is not found in culture. The *Efe-afpA* gene was expressed 700-fold greater in the leaf sheath of *E. festucae* infected strong creeping red fescue than in culture[54]. *Efe*-AfpA is found in the apoplast of endophyte infected strong creeping red fescue, but not in a high enough abundance that would suggest purification from the apoplast as being viable[8]. Therefore, this required utilizing another expression system for large scale purification of *Efe*-AfpA. The yeast *Pichia pastoris* (currently referred to as *Komagataella phaffi*[55]) is commonly used for heterologous protein expression. *Pichia pastoris* was utilized to produce *Efe*-AfpA as it had also been used to produce *A. giganteus*'s AFP[8,56,57].

Larger quantities of the protein were able to be purified from transformed *P. pastoris*, although the protein was not completely pure. Several tests were performed, all concluding that *Efe*-AfpA had antifungal activity against *C. jacksonii* by permeabilizing the membrane[8]. While active protein was obtainable, induction of expression was cumbersome requiring two different media, a daily addition of methanol, and yielded impure protein. These factors lead to the search for an optimal expression system.

Efe-AfpA was expressed in and purified from *E. coli*, *P. chrysogenum*, and *P. pastoris* and the antifungal activities were compared to PAF produced by a *P. chrysogenum* overexpressor strain[9,58]. The heterologous system used to express *Efe*-AfpA had an effect on its antifungal activity against *Neurospora crassa in vitro*, with the *P. chrysogenum* derived *Efe*-AfpA having the highest antifungal activity at relatively low concentrations. Its activity was also the most similar to that of PAF. This *Efe*-AfpA's activity against *C. jacksonii* was confirmed in culture and was assayed to determine if it could control dollar spot disease on both creeping bentgrass and endophyte-free strong creeping red fescue in a greenhouse setting. Dollar spot disease symptoms were reduced on both turfgrasses[9].

Several pieces of evidence therefore indicate that *Efe*-AfpA is a major component in the endophyte-mediated disease resistance seen in strong creeping red fescue infected by *Epichloë festucae*: (1) Uniqueness of the *Efe-afpA* gene amongst *Epichloë* spp.; (2) high levels of expression *in symbio*, but not in pure culture; (3) its antifungal activity against *C. jacksonii* in culture, and; (4) its reduction in dollar spot disease severity in greenhouse trials when applied to non-endophyte infected strong creeping red fescue inoculated with dollar spot. The next piece of evidence required to fully validate this protein's involvement in disease resistance would be a strong creeping red fescue isolate infected with an *Efe*-AfpA *E. festucae* knockout.

Two independent *Efe-afpA* knockouts were generated using CRISPR-Cas9, and neither could infect strong creeping red fescue whereas the wild type and the complemented isolates could. This suggested that the *Efe*-AfpA protein could be an effector with an additional role in the symbiotic relationship between strong creeping red fescue and *E. festucae* beyond disease resistance[54]. Other antifungal proteins have also been described to have more than one function[59,60].

Other methods could be used in lieu of a knockout such as transgenic turfgrass expressing *Efe*-AfpA. Grasses normally susceptible to dollar spot disease, such as non-endophyte infected strong creeping red fescue and creeping bentgrass, could heterologously express the antifungal protein and then be inoculated with *C. jacksonii*. Reduction in disease severity compared to an untransformed control would support *Efe*-AfpA's role in disease resistance. Both red fescue and creeping bentgrass have been shown to be amenable to transformation[61,62].

Epichloë endophytes are known to affect their hosts and produce many secondary metabolites, so other mechanisms may be involved with this disease resistance. From the transcriptomic work by Ambrose & Belanger[7] no candidate plant genes for disease resistance or defense response were

identified with the highest percentage of upregulated plant tags being in the gene ontology category 'photosynthesis'. So while *E. festucae* has an impact on the host gene expression, no obvious plant genes stood out that explained the endophyte-mediated disease resistance. *Epichloë* spp. also have an effect on the host plant's microbial community. *Festuca arundinaceum* (tall fescue) infected with *E. coenophiala* had a different fungal community than its endophyte-free counterpart, particularly in the amount of *Puccinia coronata*, a common fungal pathogen[63]. This different population may also play a role in disease resistance, but more work needs to be done to further elucidate if this is the case with strong creeping red fescue and dollar spot disease.

Recently Card, Bastìas, and Caradus excellently reviewed publications detailing the antagonism of *Epichloë* species against plant pathogens[3]. Many of these studies utilized dual culture assays where the *Epichloë* spp. were cultured with another fungus and growth inhibition was observed[64–76] while others did show antifungal activity in planta described as antibiosis[70,73–75,77–82]. Of the 29 studies cited spanning decades of work where in vitro or in planta bioactivity was reported to be due to antibiosis, only 5 identified the compounds responsible for the antifungal effect[75,76,83–85]. More recently, antifungal compounds were identified from *E. bromicola* and *Epichloë* strains NEA12 and NEA23[86,87].

Besides the highly abundant *Efe-afpA* tag, the *Epichloë* transcriptomic data identified many more small, secreted, *Epichloë* specific proteins. *E. festucae* is known to produce small secreted proteins, some of which have been predicted to be effectors from a study done on its symbiosis with *Lolium perenne*. Of the three effectors investigated, no functions were determined beyond localization in the host plant[88]. It is possible that these proteins also play a role in disease resistance or the induction of a defense response in the host. Further knockouts, overexpressors, or purifications of the other abundant *E. festucae* proteins should be conducted to further validate their functions. While many publications have reported antifungal activity by *Epichloë* spp. only a few have specifically identified the compounds involved and confirmed that the compounds are found *in symbio* as opposed to pure culture, which may not represent the compounds produced by the endophyte in the host plant. Bioprospecting these metabolites using advanced analytical methods and software should help streamline and expedite this process[89]. So, despite the other players that could be involved, the evidence gathered about *Efe*-AfpA solidifies its place as a major component of the endophyte-mediated disease resistance seen in strong creeping red fescue.

Efe-AfpA's potential mode of action was investigated, much like that of the antifungal proteins AFP and PAF. AFP from *A. giganteus* appears to rely heavily on both the presence of glycosylceramides (GlcCer) on the membrane and the unsaturation of the C-3 carbon in the glycosylceramides in the target organism. In experiments where the glucosylceramide synthase protein was inhibited, the sensitive fungus *A. niger* had decreased susceptibility. When the $\Delta(3)$ desaturase gene was knocked out in the AFP-sensitive *A. niger* it also had decreased susceptibility[90]. PAF's, from *P. chrysogenum*, mode of action was also investigated in relation to glucosylceramides with the GlcCer *N. crassa* mutants. All mutants generated in the glucosylceramide pathway, including *gcs*, lead to decreased susceptibility to PAF but not PAFB, also from *P. chrysogenum*, indicating that not only are glucosylceramides important for PAF's activity but that PAFB may have other membrane constituents with which it interacts[46]. The antifungal activities of *Efe*-AfpA and PAF were assayed against a selection of these glucosylceramide mutants. It was found that while PAF required the presence of glucosylceramide for full activity, *Efe*-AfpA was highly antifungal against all the mutants[9]. This indicated that while these two proteins share sequence and predicted structural similarities, their modes of action are different.

AFP and PAF, among other antifungal proteins, have been tested against a variety of fungi to determine their activities. This is important as these proteins could potentially represent a new type of disease control method and understanding of which can be used against which plant pathogenic fungus will be a necessity. *Efe*-AfpA was assayed against a variety of plant pathogenic fungi in addition to *C. jacksonii*. In culture *Efe*-AfpA had antifungal activity against fungi that cause economically important diseases, such as *Botrytis cinerea*, *Fusarium graminearum*, and *Pyricularia oryzae*[91].

Antifungal proteins and disease control

Management of fungal plant pathogens is a problem for many crops. Current management strategies rely heavily on the use of fungicides, which are considered critical for global food security[92,93]. Fungicides are also used heavily on amenity crops such as turfgrasses[94]. Although deemed critical for disease management, there are several problems associated with fungicide use, including toxicity to non-target organisms and development of resistance in the target fungus[92]. Another strategy for disease control is the development of alternatives or complements to synthetic fungicides with reduced toxicities. This approach can involve application of biological control organisms or products derived from living organisms to crops to reduce disease severity[95]. In particular, antifungal proteins produced by fungi are being researched for their utilization as biofungicides[96].

The efficacies of other antifungal proteins have been studied, with purified antifungal protein being applied to the plant to reduce or prevent disease. The MIC for AFP from *A. giganteus* against the rice blast fungus *Pyricularia oryzae* (formerly *Magnaporthe oryzae*) was determined to be 4 μM *in vitro*. In both detached leaf assays and whole plant experiments, treatments with AFP protected the plants from rice blast as compared to the untreated control[33]. Tomato roots that were pre-treated with AFP of different concentrations were challenged against *Fusarium oxysporum* f. sp. *lycopersici*. When pre-treated with a concentration of 100 $\mu g \cdot mL^{-1}$ of AFP, the tomato plants were protected from the pathogen while lower concentrations were not effective[49]. Reduction of barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) and wheat leaf rust (*Puccinia recondite* f. sp. *tritici*) was shown by either applying PAF from *P. chrysogenum* to leaves or floating detached leaves in a PAF solution[97]. PAFB was shown to be effective in reducing disease on oranges and apples caused by *P. digitatum, P. italicum*, and *P. expansum*, all of which are post-harvest problems on fruits[98].

The turfgrass industry in the United States is extremely lucrative. Turfgrass is the fourth largest crop in the United States with 50 million acres and an annual value of $40 – 60 billion[99]. Given how economically important it is, and how costly fungicide applications can be to control diseases such as dollar spot,

any alternatives or additives to current management programs would be very useful not only to the US but internationally as well. Since applications of purified antifungal proteins can reduce plant disease symptoms, and *Efe*-AfpA was previously shown to inhibit growth of *C. jacksonii*, the *E. festucae* antifungal protein could be a potential alternative or additive to current control measures for dollar spot disease as well as other fungal pathogens. This would require scaled up culture conditions allowing for the purification of large amounts of the protein needed for field efficacy trials. An important facet of this scale up would be the selection of an appropriate expression system as *E. festucae* does not produce *Efe*-AfpA in culture[54]. When it comes to fungicides on the market today, expression systems are not used as the fungicidal compounds are produced through chemical means. This method could be used for small antimicrobial proteins, as the production of PAF through chemical ligation was successful in producing a protein with the same structure and antifungal activity as the native PAF[100]. However, between the complicated methodologies, some of which failed, described by Varadi et al. and the ease of which many of these antifungal proteins are produced using fungal expression systems, heterologous expression is still the most efficient method[100].

When it comes to expression systems, one would want to select those that produce high levels of active protein with the least amount of economic and physical input. In other words, an expression system that is cheap and easy to use. Three expression systems were tested for the production of *Efe*-AfpA: *P. pastoris*, *E. coli*, and *P. chrysogenum*.

The *Pichia* system, while capable of producing AFP easily, yielded impure *Efe*-AfpA and was cumbersome due to multiple media types and the necessity of methanol for induction of expression[8,56]. Bacteria present an attractive alternative expression system with several marketed kits for easy transformation, growth, and heterologous protein purification. However, correct disulfide bond pairing can be difficult to achieve in heterologous systems, which can lead to mispairing, mis-folding, and protein aggregation[101]. This is even more important as it has been shown that incorrect pairing can lead to impaired function of the PAF protein. When the six cysteine residues in PAF were reduced by dithiothreitol, causing a loss of the disulfide bonds, the modified PAF no longer had any inhibitory activity against *Aspergillus niger*[32].

SHuffle cells and the Expresso® T7 SUMO Cloning and Expression System[102] were used to ensure proper folding of *Efe*-AfpA. SHuffle cells are the *E. coli* strain *trxB gor* suppressor strain, which has had its reductive pathways in the cytoplasm diminished yielding an environment primed for the oxidation of the sulfhydryl groups leading to the formation of disulfide bonds. It also overexpresses DsbC, a disulfide bond isomerase that acts as a chaperonin to further ensure correct bond formation and pairing[103,104]. However, the resulting *Efe*-AfpA had very different levels of activity compared to its *Pichia* and *Penicillium* produced counterparts and the highly similar PAF protein which may be due to structural differences involving these disulfide bonds. This system becomes even less useful because of its low yield[9].

Small cationic proteins have also been produced in a *P. chrysogenum* system developed from the ΔPAF mutant[59]. Multiple PAF and PAF-like proteins (NFAP from *Neosartorya fischeri*) have been transformed and expressed in the *P. chrysogenum* ΔPAF system yielding high quantities and biologically active proteins[43,58]. This provides an alternative to a bacterial system which may not be able to accommodate the correct pairing of disulfide bonds. The PAF expression system resulted in the highest yield of very active *Efe*-AfpA[9]. It also utilizes very common reagents for the media, does not require an additive to induce expression, and allows for a one step purification of the protein using a cation exchange resin[58]. As such, it is the most straightforward and easiest of the systems tested to use for expression and purification of active protein.

A variety of other experimentation and testing is required to further determine these antifungal proteins' efficacy in disease control. Field testing is the ultimate test, but stability studies are required to first see if these proteins can persist on plants long enough to provide protection. Heat, ion concentrations, and protease sensitivity have been investigated for some of these antimicrobial proteins[29,32,48,49], but another obvious stress to test would be light stability particularly UV sensitivity. Stability of these proteins in different formulations would also need to be examined as many chemical fungicide products are sold in a variety of forms: liquid, solids, wettable powder, etc. Should stability be a problem, adjuvants could also be added to these formulations to provide increased stability and persistence of the protein in the field. To answer these questions however, one first needs to produce large quantities of active protein, which is why expression system selection is paramount.

Conclusions

Fungal endophytes of plants have long been known to impart benefits to their host, but the exact mechanisms of which have been an ongoing area of research. Many of these benefits come from the secondary metabolites the fungal endophyte produces such as the antiherbivory alkaloids. While antagonism against a variety of fungi has been reported, very few studies verify the compounds involved and confirm their presence *in symbio*. The endophyte-mediated disease resistance imparted by *E. festucae* in strong creeping red fescue, which protects the grass host from dollar spot disease, is a more unique benefit among clavicipitaceous endophytic fungi. While this field phenomenon was observed decades ago, the molecular mechanism is only now being understood. Several lines of research have now revealed the endophyte-mediated disease resistance is likely due to an endophyte produced antifungal protein, *Efe*-AfpA, which has activity against *C. jacksonii*. *Efe*-AfpA, and other antifungal proteins like it such as AFP and PAF, has activity against many fungi and could represent a new mechanism for disease control. Further studies are required to fully understand *Efe*-AfpA's efficacy against dollar spot disease, specifically scale up, UV stability, and field trials. In tandem, research into its mode of action should be continued. Once fully characterized, *Efe*-AfpA and other proteins like it could represent a new product for plant disease control alleviating the use of chemical fungicides resulting in less environmental impacts and a reduction in the selection for resistant fungal pathogens.

Author contributions

The author confirms sole responsibility for manuscript preparation.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Conflict of interest

The funders of the previous research mentioned above had no role in the writing of this manuscript. The author declares he has no conflict of interest. Rutgers University has filed a provisional patent concerning the purification and use of *Efe*-AfpA for fungal pathogen disease control.

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