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REVIEW

Proteases from phytopathogenic fungi and their importance in phytopathogenicity

Murugesan Chandrasekaran[1] · Boopathi Thangavelu[2] · Se Chul Chun[1] · Muthukrishnan Sathiyabama[3]

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Abstract Phytopathogenic fungi, causal agents of some of the world's most serious plant diseases, can significantly reduce yields during large-scale agricultural production. Among the numerous hydrolytic enzymes they produce for nutritional and/or pathogenicity purposes, hydrolases and proteases are required for their growth and survival. The present review focuses on extracellular and/or secretory proteases from phytopathogenic fungi. Several extracellular proteases have been identified that contribute to fungal growth, infection structure formation, cell wall degradation, proteolytic processing of pathogenesis-related proteins and that act as elicitors of defense responses. In this review, the positive correlation between protease secretion and disease aggressiveness and/or necrosis is highlighted. The involvement of various fungal proteases in pathogenic mechanisms makes them potential targets for designing protease inhibitors that may provide an improved way to combat plant diseases, which in turn will reduce dependence on fungicides.

Keywords Proteases · Serine proteases · Metalloproteases · Phytopathogenic fungi · Phytopathogenicity · Virulence factors

✉ Muthukrishnan Sathiyabama
sathiyabamam@yahoo.com

[1] Department of Bioresource and Food Science, Konkuk University, Seoul, Republic of Korea

[2] Department of Marine Biotechnology, Bharathidasan University, Tiruchirappalli, Tamilnadu, India

[3] Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Introduction

Phytopathogenic fungi are a serious threat to plant health, causing a plethora of diseases that contribute substantially to overall losses in agricultural yield. In addition, ~10 % of all known fungal species can cause plant disease, affecting more than 10,000 plant species (Horbach et al. 2011; Kubicek et al. 2014). Cell-wall-degrading enzymes are important for determining the ability of a pathogen to colonize a host plant (Annis and Goodwin 1997; Juge 2006; Lebeda et al. 2001; Nakajima and Akutsu 2014). There is also considerable evidence implicating pectinases (Annis and Goodwin 1997; Nakajima and Akutsu 2014) and to a lesser extent cellulases (Doi and Kosugi 2004) and xylanases as virulence factors (Annis and Goodwin 1997; Nakajima and Akutsu 2014). Initially, when pathogenic fungi encounter a host, they produce various cell-wall-degrading enzymes such as glycanases and proteases to fragment the plant cell wall polymers, thus facilitating penetration into the host cells (Chu et al. 2015; Doi and Kosugi 2004; Kubicek et al. 2014; Jashni et al. 2015a). Apart from their role in cell wall degradation, proteases synthesized by plant pathogenic fungi have also been proposed as possible virulence factors in plant–pathogen interactions (Jashni et al. 2015a; Slavokhotova et al. 2014) (Table 1). Moreover, plant cell walls possess several proteins, glycoproteins, and structural proteins. Therefore, proteolytic enzymes play an important role in pathogenesis (Jashni et al. 2015b; Saitoh et al. 2009). However, most saprophytic fungi produce proteolytic enzymes for their nutritional requirements. Thus, the type and level of protease production determine the saprophytic or pathogenic characteristics of fungi. The role of these enzymes in the process of pathogenesis has been the subject of intensive research. This review aims to discuss the various features

Table 1 Proteases from plant pathogenic fungi

Pathogen	Host	Disease name	Proteolytic enzyme	References
Alternaria solani	Potato and tomato	Early blight	Serine protease Metalloprotease	Chandrasekaran et al. (2014, 2016)
Alternaria alternata	Buckwheat	Buckwheat seed diseases	Subtilisin and Trypsin-like serine proteases	Dunaevsky et al. (2006)
Aspergillus flavus	Cotton	Cotton boll infection	Extracellular protease	Brown et al. (2001)
Bipolaris zeicola	Maize	Ear rot	Trypsin-like serine protease	Murphy and Walton (1996)
Botrytis cinerea	Carrot	Carrot disease	Aspartic protease	Movahedi and Heale (1990), Rolland et al. (2009) and ten Have et al. (2010)
Colletotrichum coccodes	Tomato	Brown root rot	Serine protease	Redman and Rodriguez (2002)
Fusarium oxysporum	Tomato	Vascular wilt	Serine, cysteine and metallo protease	Jashni et al. (2015b)
Fusarium solani	Potato	Dry rot	Serine protease	Olivieri et al. (2002, 2004)
Fusarium solani f. sp. *eumartii*	Potato	Dry rot	Extracellular protease	Olivieri et al. (2004)
Fusarium sporotrichioides	Rye	Ear fusariosis	Trypsin-like serine protease	Dunaevsky et al. (2008)
Fusarium verticillioides	Maize	Stalk and ear rot	Metalloprotease	Slavokhotova et al. (2014)
Gibberella gordonii	Wheat	Root rot in wheat	Trypsin-like serine protease	Dunaevsky et al. (2008)
Glomerella acutata	Apple	Soft rot in fruits	Aspartic, cysteine and serine protease	Gregori et al. (2010)
Magnaporthiopsis poae	Kentucky bluegrass	Root rot	Subtilisin-like serine protease	Sreedhar et al. (1999)
Neocosmospora haematococca	Pea	Root rot	Trypsin-like serine protease	St. Leger et al. (1997)
Penicillium chrysogenum *Penicillium glabrum*	Strawberry	Fruit rot	Subtilisin-like serine protease	Dunaevsky et al. (2006)
Peronospora hyoscyami f. sp. *tabacina*, *Phytophthora nicotianae*	Tobacco	Black shank	Serine protease	Silva et al. (2013)
Pleurotus pulmonarius	Wood	Wood rot	Subtilisin-like serine protease	Kudryavtseva et al. (2008)
Pleurotus ostreatus	Wood	Wood rot	Trypsin-like serine protease	Inácio et al. (2015)
Phytophthora infestans	Potato	Late blight	Serine protease	Feldman et al. (2014)
Phytophthora infestans, *Thanatephorus cucumeris*, *Fusarium culmorum*	Potato	Potato disease	Serine protease	Kudryavtseva et al. (2013), Pekkarinen et al. (2002) and Valueva et al. (2011)
Pyrenopeziza brassicae	Oil seed rape	Leaf spot	Cysteine protease	Ball et al. (1991)
Pyricularia grisea, *Nakataea oryzae*	Rice	Rice blast	Metallo protease and subtilisin-like protease	Jia et al. (2000) and Saitoh et al. (2009)
Sarocladium strictum	Strawberry	Fruit rot and Wilt	Subtilisin-like serine protease	Chalfoun et al. (2013)
Sclerotinia sclerotiorum	Common Bean	White mold	Non-aspartyl acid protease	Poussereau et al. (2001)
Sphacelotheca reiliana	Corn	Head smut	Aspartyl acid protease	Mandujano-González et al. (2013)
Ustilago maydis	Corn	Smut	Non-aspartyl acid protease	Mercado-Flores et al. (2003)
Verticillium alboatrum, *Verticillium dahliae*	Tomato	Tomato wilt	Serine protease	St. Leger et al. (1997)
Verticillium dahliae	Cotton	Vascular wilt	Serine protease	He et al. (2015)

of proteases secreted by phytopathogenic fungi and their role in virulence.

Classifications of proteases

Proteases are one of the most important industrial enzymes, accounting for nearly 65 % of the total worldwide enzyme sales (Kasana et al. 2011; Souza et al. 2015). The protease (E.C. 3.4) group of enzymes hydrolyzes peptide bonds [Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)]. Proteases are classified based on their hydrolytic process (endopeptidases and exopeptidases), pH (acidic, alkaline and neutral proteases), or functional group at the active site (aspartic, cysteine, glutamic, metallo, serine, threonine, and unknown (NC-IUBMB). According to the MEROPS peptide database, proteolytic enzymes are grouped into 12 clans and 35 families based on amino acid sequence similarity and catalytic mechanism (Rawlings et al. 2012). Most of the fungal proteolytic enzymes are represented largely by serine proteases, named for the nucleophilic Ser residue at the active site (Asp-His-Ser). Based on their substrate specificity, they were divided into two major families of serine-like proteases such as trypsin-like (TLPs) and subtilisin-like proteases (SLPs) (Rawlings et al. 2012). The specificity of TLPs is restricted to the C-terminal side positively charged residues (Lys and Arg) because of the Asp 189 residue located in the binding pocket. SLPs cleave peptide bonds located on the C-terminal side of large hydrophobic residues (Phe, Trp, Met, Tyr and Leu) as a result of the hydrophobic makeup of the pocket (Rawlings et al. 2012). Moreover, enzyme activity has been determined using synthetic substrates. It is evident that *Phytophthora infestans* exoproteinases most effectively hydrolyze *N*-α-benzoyl-L-Arg-pNa (BAPNA) (a substrate for TLPs) and to a lesser extent *N*-carbobenzyloxy-L-Ala-L-Ala-L-Leu-pNa (Z-AALPNA) (a substrate for SLPs). At the same time, exoproteinases did not act on the substrates for chymotrypsin-and elastase-like proteinases (*N*-succinyl-glycyl–glycyl-L-phenylalanine *p*-nitroanilide [Suc-GGFPNA] and *N*-acetyl-L-alanyl-L-alanyl-L-alanyl *p*-nitroanilide [Ac-AAAPNA], respectively), nor did aminopeptidases [L-leucine *p*-nitroanilide (LPNA)]. The enzymes secreted by *Fusarium culmorum* hydrolyze Z-AALPNA very efficiently and to a lesser extent BAPNA. They have low activity toward substrates for chymotrypsin-like and elastase-like proteinases and for aminopeptidases (Valueva et al. 2011, 2015). Thus, the proteases from phytopathogenic fungi have unique properties and deserve further study.

Protease production and phytopathogenicity

Phytopathogenic fungal proteases can be produced in various conditions and ways. Depending on culture conditions, different forms of the same protease can be expressed (Inácio et al. 2015). Many species of phytopathogenic fungi produce different proteases in a variety of culture media and host tissues (Chandrasekaran et al. 2014; He et al. 2015; Valueva et al. 2015). Casein is a traditional substrate used to determine proteolytic activity, and hemoglobin, rennin, azo-casein, serum albumin, gelatin, yeast and other proteinaceous substrates have been used to a lesser degree (Chandrasekaran et al. 2014; Valueva et al. 2011, 2015). According to Zaferanloo et al. (2014), the optimum condition for protease production of *Alternaria alternata* is 37 °C, pH 7.0 using soybean as substrate. The optimum condition for protease production in *Alternaria solani* is 27 °C, pH 8.5 with casein as the substrate (Chandrasekaran and Sathiyabama 2014; Chandrasekaran et al. 2014, 2016). The addition of heat-stable potato tuber proteins to the culture medium is vital for secretion of exoprotease from *Thanatephorus cucumeris* and *F. culmorum* (Valueva et al. 2011). Recently, Mandujano-González et al. (2013) identified the 41-kDa extracellular aspartyl protease Eap1 from *Sphacelotheca reiliana* in both solid and liquid culture media. Similarly, in *Ustilago maydis*, non-aspartyl acid extracellular protease pumAe was found under acidic conditions in the culture medium (Mercado-Flores et al. 2003). According to Feldman et al. (2014), the oomycete *P. infestans* secretes serine and metalloproteases into culture media; metalloproteases are more abundant. These studies suggest that different nutritional sources are essential for the differential production of various proteases.

Fungal proteases are thought to be important during different aspects of the infection process, including adhesion to host cells, initial penetration of the plant cell wall and colonization (Brown et al. 2001; Movahedi and Heale 1990; Olivieri et al. 2004; Soberanes-Gutiérrez et al. 2015). A correlation between proteolytic activity in the culture medium and in the infected plant has been shown for several phytopathogenic fungi to establish disease (Bindschedler et al. 2003; Pekkarinen et al. 2002). According to Movahedi and Heale (1990), 38–39 kDa aspartyl acid proteases were detected in the culture medium of *Botrytis cinerea* and in carrot tissue infected by the fungus, but not in uninfected carrot tissue. Further, cell death investigations by Movahedi and Heale (1990) for *B. cinerea* suggested that aspartic protease might be the most important factor contributing to the initial stage of infection. Significant extracellular proteolytic activity in Spunta potato tubers during infection by *Fusarium solani* (Olivieri et al.

2004) confirms proteases as a key regulator of infection and immunity-priming factor in plant hosts. An investigation of the genetic basis of proteolytic activity in *Sclerotinia sclerotiorum* showed the expression of genes *acp1* and *asps* responsible for the production of acid protease and aspartyl protease, respectively (Poussereau et al. 2001). In a similar study, Rolland et al. (2009) identified BcACP1, a member of the G1 family of protease, produced by *B. cinerea* during infection. An array of proteases such as aspartic proteases, cysteine proteases, and serine proteases were produced by *Glomerella acutata* (a causal agent of soft rot in fruits) (Gregori et al. 2010), showing the involvement of several proteases in disease development. Recently, ten Have et al. (2010) demonstrated that aspartic proteases are the key factors of pathogenicity in *B. cinerea*. It is clear from recent studies of several phytopathogenic fungi that the importance of proteases in the disease process may depend upon the specific plant–pathogen interaction (Figueiredo et al. 2014; Jashni et al. 2015a; Soberanes-Gutiérrez et al. 2015; ten Have et al. 2010).

Furthermore, the necessity of extracellular proteases in phytopathogenicity was confirmed by mutational studies in several fungal species such as *Pyrenopeziza brassicae*, *Verticillium dahliae*, *Nakataea oryzae*, *U. maydis* and *F. oxysporum* (Ball et al. 1991; Dobinson et al. 2004; Saitoh et al. 2009; Jashni et al. 2015a; Soberanes-Gutiérrez et al. 2015). An extracellular protease was reported to be a significant pathogenicity factor of *Colletotrichum coccodes*, and its removal by mutagenesis converts a virulent pathogen into a nonpathogenic endophyte (Redman and Rodriguez 2002), while in *Bipolaris zeicola* disruption of the *ALP1* gene, which encodes a TLP, had no distinct effect on virulence (Murphy and Walton 1996). Saitoh et al. (2009) confirmed that that the *SPM1*-encoded SLP is a major protease for autophagy that might affect endocytosis during pathogenesis of the rice blast fungus *N. oryzae*.

The secretion of protease inhibitors (PI) during plant–pathogen interactions implies the existence of protein targets in the fungal pathogens. The lack of protease activity in phytopathogenic fungus correlates with the lack of proteases in most fungi. Transient expression of serine protease inhibitor gene *NmIMSP* in susceptible tobacco leads to enhanced resistance to the oomycetes *Peronospora hyoscyami* f. sp. *tabacina* and the *Phytophthora nicotianae* (Silva et al. 2013). A PI mixture obtained from potato sprouts is strongly inhibitory to proteases, spore germination, hyphal elongation and necrotic activity of *B. cinerea* (Turra 2006). Moreover, PLPKI (potato serine protease inhibitor I family) inhibited the activity of proteases secreted by two pathogens of potato, the oomycete *P. infestans* and the fungus *Thanatephorus cucumeris*, but not of the proteases of nonpathogenic *Thanatephorus* N2. Furthermore, a clear correlation between PLPKI activity and the degree of horizontal resistance against *P. infestans* was corroborated on potato clones at varying degrees of resistance to this oomycete. The fact that plants have developed ways to sense proteases (either directly or through the products of their actions) highlights their importance in pathogenesis. In response to perception, a series of signalling events takes place (involving protease inhibitors, antimicrobial peptides, PR proteins, mitogen-activated protein kinase [MAPK] signaling, and interactions between the products of plant resistance [*R*] and pathogen avirulence [*avr*] genes), that is shared among various elicitors and eventually leads to the deployment of defence mechanisms, again implicating proteases in pathogenicity (Cheng et al. 2015; Ryan 1990; Slavokhotova et al. 2014).

Role of proteases in plant–fungi interactions

Table 1 lists phytopathogenic fungi that secrete a broad spectrum of proteolytic enzymes during penetration and colonization of host tissues. Proteolysis, a vital process for cell life, plays an important role in different physiological functions such as post-secretion protein processing, germination, sporulation, aerial mycelium and appressorium formation, nutrition, and adaptation to different environmental conditions (Chandrasekaran and Sathiyabama 2014; Tucker and Talbot 2001). In plants, fungal proteolytic mechanisms are responsible for removal of abnormal or nonfunctional proteins, activation/inactivation of specific proteins and autolytic processes. In addition, proteases increase the permeability of the plant plasma membrane, suggesting that they may have a critical role in phytopathogenesis (Soberanes-Gutiérrez et al. 2015). Polypeptides released upon the action of proteases secreted from phytopathogenic fungi may act as elicitors, (damage-associated molecular patterns), which are subsequently recognized by corresponding immune receptors and are known to trigger MAPK signaling. A metalloprotease from the rice blast fungus *Pyricularia grisea* (now *P. oryzae*) was shown to act as an avirulence factor (AVR-Pita) by directly binding to a plant resistance gene product (Pi-ta) and stimulating a signaling cascade that leads to resistance in a classic example of a gene-for-gene interaction (Jia et al. 2000). Olivieri et al. (2002) demonstrated that the fungus *F. solani* f. sp. *eumartii* secretes 30 kDa SLP with the ability to degrade pathogenesis related (PR) proteins as well as specific polypeptides of intercellular washing fluids and cell wall proteins. A study on a novel extracellular elicitor protein produced by the strawberry pathogen *Sarocladium strictum* confirmed that the proteolytic activity of the AsES elicitor is required for the induction of defense responses in plants (Chalfoun et al. 2013). Other

reports suggest that pathogens might overcome the deleterious effects of plant chitinases by secreting proteases that modify them. A recently discovered Zn-metalloprotease from *Fusarium verticillioides*, fungalysin Fv-cmp, cleaves the Gly–Cys peptide bond between two domains of corn class IV chitinases ChitA and ChitB (Slavokhotova et al. 2014). Fungalysin-mediated proteolytic degradation of class IV chitinases is also found in *Arabidopsis* (Naumann and Price 2012).

Proteases as markers of phytopathogenicity

Positive correlations between certain protease activities and disease aggressiveness have been reported in several plant pathogenic fungi. A cerevisin (homologous to proteinase B from *S. cerevisiae*) mutant of *V. dahliae* with decreased secretion of low molecular weight proteins known to be virulence factors was generated to assess the effects on virulence. At 21 days after inoculation with the cerevisin mutant, cotton seedlings did not have noticeable symptoms. Even though the role of cerevisin in the production and secretion of these polypeptides remains to be elucidated, the experiments highlight the indirect involvement of cerevisin in the virulence of *V. dahliae* (He et al. 2015). Soberanes-Gutiérrez et al. (2015) reported that the knockout of the *PEP4* gene in *U. maydis* reduced virulence of *U. maydis* on maize. Nonpathogenic mutants of the fungus *Pyrenopeziza brassicae* do not have the ability to produce extracellular cysteine proteinase (Ball et al. 1991). Staples and Mayer (1995) showed that an increase in the level of aspartic protease activity in *B. cinerea* led to an increase in virulence. *Magnaporthiopsis poae* expresses a SLP (Mp1) in infected roots, and its level of expression is positively correlated with the severity of symptoms (Sreedhar et al. 1999). Olivieri et al. (2004) demonstrated a correlation between proteolytic activity detected in intercellular washings with the size of lesions caused by *F. eumartii* in susceptible potato tubers, but not in the resistant. Higher proteolytic activity in roots infected with *F. solani* f. sp. *phaseoli* correlated with the processing of class IV chitinase (Lange et al. 1996). Recently, Jashni et al. (2015b) reported that both secreted metalloprotease and a serine protease were responsible for reduced chitinase activity in tomato. Because these chitinases have antifungal properties, the deletion of both encoding genes (FoMep1 and FoSep1) rendered *F. oxysporum* f. sp. *lycopersici* unable to cleave and inactivate specific chitinases and thus less virulent on tomato.

Dunaevsky et al. (2001, 2006) reported that the production of TLPs is characteristic of plant pathogens. Further, the validity of TLPs as a marker in fungal phytopathogenicity was proved to be due to conserved motifs around the active site residues (Dubovenko et al. 2010). Nevertheless, SLPs are the key virulence factors in nematophagy (Zhao et al. 2005) and responsible for pathogenesis in saprophytes such as *A. solani* (Valueva et al. 2013). In the plant pathogens *V. albo-atrum*, *V. dahliae*, and *Neocosmospora haematococca*, the protease activities detected were trypsin-like, with only trace levels of SLP (St. Leger et al. 1997). A comparative study of extracellular proteases of six species of mycelial fungi, including three phytopathogens (*A. alternata*, *B. cinerea*, and *Alternaria botrytis*) and three saprophytes (*Penicillium chrysogenum*, *Penicillium glabrum* and *Trichoderma harzianum*) showed that SLPs are likely present in all surveyed fungal species (Dunaevsky et al. 2006). More virulent *F. sporotrichioides* exhibited greater extracellular TLP activity than the less virulent *Gibberella gordonii* (Dunaevsky et al. 2008). Some fungi such as *T. harzianum* and other saprotrophic fungi secrete solely SLPs. However, *A. alternata* and other phytopathogens produce TLPs as well (Valueva et al. 2015). Kudryavtseva et al. (2008, 2013) found that *Pleurotus pulmonarius*, which grows on dead timber, secretes subtilisin but not trypsin. *Pleurotus ostreatus*, which grows in living hosts, secretes extracellular trypsin throughout its development, suggesting that successful colonization of living tissues requires TLP (Inácio et al. 2015). By using SLPs, a pathogenic fungus mainly affects the physiological integrity of host during penetration and colonization (Chalfoun et al. 2013; Dunaevsky et al. 2006; Figueiredo et al. 2014). There is evidence of extensive gene duplication and loss within the SLP family in fungal lineages, which has been correlated with differences in fungal lifestyles. These data indicate that the production of TLPs is a distinct feature of phytopathogenic fungi, whereas the production of SLPs is a specific feature of saprotrophic fungi (Dubovenko et al. 2010; Kudryavtseva et al. 2008). In some fungi, SLPs either can play a general nutritive role, or may play specific roles in cell metabolism or as pathogenicity or virulence factors. Thus, the presence of significant extracellular TLPs and/or SLPs activity in phytopathogenic fungi may be considered as an indicator of their phytopathogenicity.

Conclusion and future perspectives

In this review, the role in pathogenicity of proteases from phytopathogenic fungi was discussed, including during host–pathogen interactions and signaling involved in priming an immune response and the fact that the energy requirements of phytopathogens are met after the degradation of plant cell wall proteins. Identification and characterization of fungal proteases will be a key aspect in the generation of new molecular markers for phytopathogenicity. Isolation and analysis of

genes that encode proteases will promote further investigation of their genetics, regulation, and evolution. In the case of redundant gene families, studies on multiple protease mutants will be needed to characterize their role in virulence. Advanced transcriptome and proteome analyses will facilitate identification of important proteases and their specific inhibitors for further functional analysis. Progress in this research area will allow plant pathologists to design more efficient strategies to generate pathogen-resistant plants. Metabolomics and expression profiling will help to identify specific targets of fungal proteases important in pathogenesis, thus opening new paths for the development of more resistant crops and the progress of sustainable agricultural practices.

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