A Second Kazal-Like Protease Inhibitor from *Phytophthora infestans* Inhibits and Interacts with the Apoplastic Pathogenesis-Related Protease P69B of Tomato[1]

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The plant apoplast forms a protease-rich environment in which proteases are integral components of the plant defense response. Plant pathogenic oomycetes, such as the potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) pathogen *Phytophthora infestans*, secrete a diverse family of serine protease inhibitors of the Kazal family. Among these, the two-domain EPI1 protein was shown to inhibit and interact with the pathogenesis-related protein P69B subtilase of tomato and was implicated in counter-defense. Here, we describe and functionally characterize a second extracellular protease inhibitor, EPI10, from *P. infestans*. EPI10 contains three Kazal-like domains, one of which was predicted to be an efficient inhibitor of subtilisin A by an additivity-based sequence to reactivity algorithm (Laskowski algorithm). The *epi10* gene was up-regulated during infection of tomato, suggesting a potential role during pathogenesis. Recombinant EPI10 specifically inhibited subtilisin A among the major serine proteases, and inhibited and interacted with P69B subtilase of tomato. The finding that *P. infestans* evolved two distinct and structurally divergent protease inhibitors to target the same plant protease suggests that inhibition of P69B could be an important infection mechanism for this pathogen.

The plant apoplast forms a protease-rich environment in which proteases are integral components of the plant defense response (Tornero et al., 1997; Jorda et al., 1999; van Loon and van Strien, 1999; Kruger et al., 2002; Xia et al., 2004). For example, the subtilisin-like Ser protease P69B, an apoplastic pathogenesis-related (PR) protein of tomato (*Lycopersicon esculentum*), has long been tied to plant defense (Tornero et al., 1997; Zhao et al., 2003; Tian et al., 2004). P69B is induced by multiple plant pathogens, including the oomycete *Phytophthora infestans*, citrus exocortis viroid, and the bacterium *Pseudomonas syringae* (Tornero et al., 1997; Zhao et al., 2003; Tian et al., 2004), and appears to account for a significant portion of the increase in total endoprotease activity observed in defense-activated tomato (Tornero et al., 1997; Zhao et al., 2003; Tian et al., 2004). Rcr3, an apoplastic papain-like Cys protease from tomato, is required for specific resistance to the plant pathogenic fungus *Cladosporium fulvum* (Kruger et al., 2002). In Arabidopsis (*Arabidopsis thaliana*), the extracellular aspartic protease CDR1 functions in disease resistance signaling as a positive regulator of cell death (Xia et al., 2004). These findings suggest that suppression of plant protease-mediated host defenses could be one of the diverse strategies that plant pathogens have evolved to survive in the plant intercellular space and colonize plant tissue. Indeed, our laboratory recently reported that plant pathogenic oomycetes secrete a diverse family of Kazal-like Ser protease inhibitors with at least 35 members identified from *P. infestans*, *Phytophthora sojae*, *Phytophthora ramorum*, *Phytophthora brassicae*, and the downy mildew *Plasmopara halstedii* (Tian et al., 2004). Among these, the two-domain EPI1 protein of *P. infestans* was found to inhibit and interact with tomato P69B subtilase (Tian et al., 2004). Protease inhibitors might be ubiquitous among eukaryotic plant pathogens. Avr2, a small secreted peptide of the fungus *C. fulvum*, was shown to inhibit tomato Cys protease Rcr3 (J.D. Jones, personal communication). Therefore, inhibition of plant proteases by secreted plant pathogen proteins might represent a novel counter-defense mechanism.

P. infestans causes late blight, a reemerging and ravaging disease of potato (*Solanum tuberosum*) and tomato (Birch and Whisson, 2001; Smart and Fry, 2001; Ristaino, 2002; Shattock, 2002). *P. infestans* belongs to the oomycetes, a group of fungus-like organisms that are distantly related to fungi but closely related to brown algae and diatoms in the Stramenopiles (Sogin and Silberman, 1998; Margulis and Schwartz, 2000; Kamoun, 2003). *P. infestans* is a hemibiotrophic pathogen that requires living cells to establish a successful infection. As with other biotrophic plant pathogens,

[1] This work was supported by the U.S. Department of Agriculture-National Research Initiative (project no. OHO00963–SS) and by the National Science Foundation (Plant Genome grant no. DBI–0211659). Salaries and research support were also provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.061226.

processes associated with *P. infestans* pathogenesis are thought to include the suppression of host defense responses. For example, water-soluble glucans of *P. infestans* were shown to suppress defense responses (Sanchez et al., 1992; Yoshioka et al., 1995; Andreu et al., 1998). The recent finding that the *P. infestans* Kazal-like extracellular protease inhibitor EPI1 targets tomato P69B subtilase suggests a distinct counter-defense mechanism (Tian et al., 2004). Fourteen Kazal-like extracellular Ser protease inhibitors (EPI1–EPI14) from *P. infestans* form a diverse family of secreted proteins (Tian et al., 2004). These EPI proteins were predicted to be Kazal-like inhibitors based on conserved Kazal domain motifs in their amino acid sequence. The number of Kazal domains for each EPI protein ranges from one to three. Individual Kazal domains from a multidomain inhibitor can be effective against different Ser proteases (Scott et al., 1987; Mitsudo et al., 2003; Mende et al., 2004). Therefore, it is likely that multidomain EPI proteins are able to inhibit multiple different Ser proteases. With such a diverse family of secreted protease inhibitors in *P. infestans*, complex inhibition of a diverse array of plant proteases might occur at the plant-microbe interaction interface. The same EPI protein might target different plant proteases, while different EPI proteins could also target the same plant protease.

Ser proteinase inhibitors can be classified into at least 18 nonhomologous families according to their sequences, disulfide pattern, and the three-dimensional structures (Laskowski and Qasim, 2000). The Kazal family (MEROPS family I1, http://merops.sanger.ac.uk) was named after L. Kazal, who discovered the pancreatic secretory trypsin inhibitor PSTI (Lu et al., 2001). Besides oomycetes, Ser protease inhibitors of the Kazal family are widely distributed in animals and apicomplexans. They are thought to play important roles in maintenance of normal cellular and physiological processes of animals (Magert et al., 2002; Kreutzmann et al., 2004) and pathogenesis of mammalian parasitic apicomplexans (Pszenny et al., 2000, 2002; Morris et al., 2004). As a result of exhaustive biochemical structure function analyses of the third domain of turkey ovomucoid protein conducted by the late Michael Laskowski Jr. and collaborators (Laskowski et al., 1987; Lu et al., 1997, 2001), much is known about the relationship between domain sequence and inhibition specificity in Kazal protein-Ser protease interactions. This work culminated in the development of an additivity-based sequence to reactivity algorithm, referred from here on as the Laskowski algorithm, that predicts the inhibition constants (K_i) between Kazal domains and a set of six Ser proteases based solely on the sequence of the inhibitors (Lu et al., 2001). For each Kazal domain, there are 12 contact positions (P6, P5, P4, P3, P2, P1, P1′, P2′, P3′, P14′, P15′, and P18′) responsible for the interactions between Kazal domains and the cognate Ser proteases (Read et al., 1983; Laskowski et al., 1987; Lu et al., 1997, 2001). Changes in noncontact residues typically do not affect equilibrium constants (K_a, the reciprocal of K_i), whereas changes in contact residues cause significant alterations of K_a (Lu et al., 2001). Among the 12 contact residues, P3 and P15′ are represented by conserved Cys and Asn, respectively, which show little variation in naturally occurring Kazal domains. However, the remaining 10 contact residues are hypervariable and account for inhibition specificity (Lu et al., 2001). The Laskowski algorithm was established based on mutational analyses of the 10 hypervariable contact residues and allows for the calculation of K_a or K_i of a Kazal domain against a selected set of six Ser proteases based on the domain sequence alone (Lu et al., 2001).

Subtilin-like Ser proteases (subtilases, S8 family based on MEROPS peptidase database, http://merops.sanger.ac.uk) are widely distributed in plants. In Arabidopsis, 54 subtilase genes were found (Beers et al., 2004). In tomato, at least 15 subtilase genes are known (Meichtry et al., 1999). Tomato P69 subtilases represent six different protein isoforms (P69A-F) of approximately 69 kD that accumulate extracellularly and show a high degree of similarity among each other (71%–88% identical; Jorda et al., 2000). The *P69* genes are differentially regulated by developmental and environmental signals in a tissue-specific manner (Jorda et al., 1999, 2000). The expression of *P69B* and *P69C* is induced by pathogen infection and salicylic acid (SA). The other four *P69* genes are not pathogen- or SA-induced, but they display diverse expression patterns in different organs of the plant. *P69A* is expressed in all organs except roots and flowers, while *P69E* is expressed only in roots, *P69D* is expressed in both flowers and leaves, and *P69F* is expressed only in hydathodes. The biological functions of these *P69* genes remain largely unknown, except for the possible roles of *P69B* and *P69C* in plant defense.

In this paper, we describe and functionally characterize the *P. infestans* Kazal-like extracellular Ser protease inhibitor, EPI10. EPI10 contains three Kazal-like domains, one of which was predicted to be an efficient inhibitor of subtilisin A by the Laskowski algorithm (Lu et al., 2001). The *epi10* gene was up-regulated during infection of tomato suggesting a potential role during pathogenesis. Recombinant EPI10 (rEPI10) specifically inhibited subtilisin A among the major Ser proteases, and inhibited and interacted with the PR subtilase P69B of tomato. The finding that *P. infestans* evolved two distinct and structurally divergent protease inhibitors to target the same plant protease suggests that inhibition of P69B could be an important infection mechanism for this pathogen.

RESULTS

epi10 Is Predicted to Encode an Inhibitor of Subtilisin A and Is Up-Regulated during Infection of Tomato by *P. infestans*

Previously, we used data mining of expressed sequence tags and random genomic sequence of

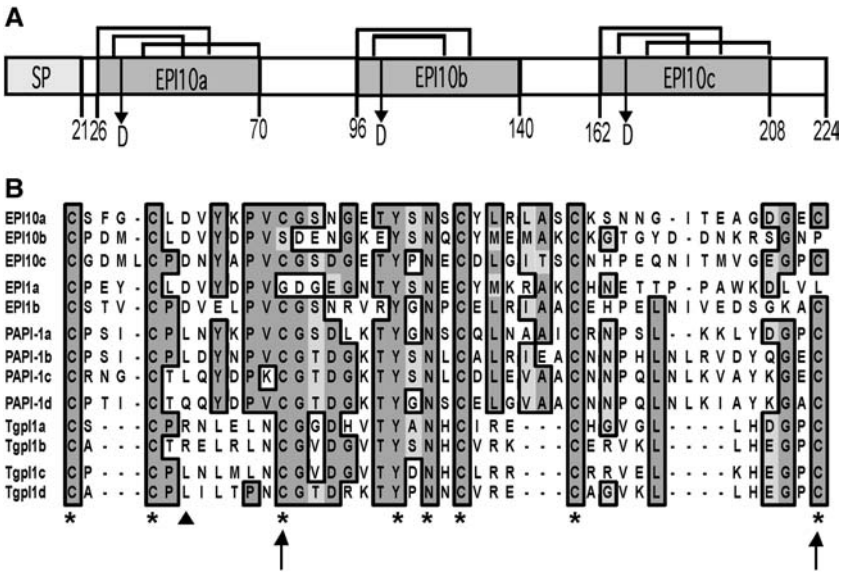

Figure 1. EPI10 belongs to the Kazal family of Ser protease inhibitors. A, Schematic representation of EPI10 structure. The signal peptide (SP) and three Kazal domains (EPI10a, EPI10b, and EPI10c) are shown in gray. Numbers indicate positions of amino acid residues starting from the N terminus. The putative disulfide linkages formed by Cys residues within the predicted Kazal domains are shown. The positions of the P1 residues D (Asp) are indicated by arrows. B, Sequence alignment of EPI domains with representative Kazal family inhibitor domains. Protein names correspond to protease inhibitors of *P. infestans* EPI1 (EPI1a-b, AY586273), EPI10 (EPI10a-c, AY586282), the crayfish *P. leniusculus* (PAPI-1a-d, CAA56043), and the apicomplexan *T. gondii* (TgPI-1a-d, AF121778). Amino acid residues that define the Kazal family protease inhibitor domain are marked with asterisks. The predicted P1 residues are shown by the arrowhead. The positions of the Cys residues that are missing in EPI10b and EPI1a are shown by arrows.

P. infestans to identify 14 Kazal-like extracellular Ser protease inhibitors (EPI1–EPI14; Tian et al., 2004). One of these proteins, the two-domain EPI1, was shown to inhibit and interact with subtilisin-like proteases, including tomato PR P69B. To identify additional subtilisin inhibitors, we used the Laskowski algorithm (Lu et al., 2001) to predict the K_i of *P. infestans* Kazal domains against subtilisin A. Of the 17 EPI domains that could be assessed with the algorithm, the first domain of EPI1 (EPI1a) and the second domain of EPI10 (EPI10b) were the only domains predicted to inhibit subtilisin A with a K_i lower than 10^{-9} M.

epi10 was first identified from an expressed sequence tag generated from zoospores of *P. infestans* 88069 (Tian et al., 2004). DNA sequencing of the full cDNA revealed an open reading frame of 675 bp corresponding to a predicted translated product of 224 amino acids, and the sequence was deposited in National Center for Biotechnology Information GenBank under accession number AY586282. SignalP 3.0 (Bendtsen et al., 2004) analysis of the putative protein identified a 21-amino acid signal peptide with a significant mean S value of 0.939. Searches against InterPro database (http://www.ebi.ac.uk/InterProScan) confirmed the presence of three domains with similarity to Kazal inhibitors (InterPro IPR002350; Fig. 1A). We used ClustalX (Thompson et al., 1997) to generate a multiple alignment of Kazal domains from *P. infestans* EPI1, EPI10, the signal crayfish *Pacifastacus leniusculus* Kazal inhibitor PAPI-1 (Johansson et al., 1994), and the apicomplexan *Toxoplasma gondii* TgPI1 (Pszenny et al., 2000; Fig. 1B). The first domain (EPI10a) and the third domain (EPI10c) of EPI10 are typical Kazal domains, containing all the highly conserved amino acid residues defining the Kazal family signature, including six Cys residues forming a 1-5/2-4/3-6 disulfide bond pattern, Tyr, and Asn residues. Like the first domain (EPI1a) of EPI1, the second domain (EPI10b) of EPI10 is atypical and lacks the third and sixth Cys but retains the other four Cys (Fig. 1). For all three domains of EPI10, the predicted P1 residues, which are central to the specificity of Kazal inhibitors (Laskowski and Kato, 1980; Komiyama et al., 1991; Lu et al., 1997), are Asp (Asp) similar to the domains of EPI1.

To assess whether *epi10* is relevant to host interaction like EPI1, we examined the expression of the *epi10* gene during infection of tomato by semiquantitative reverse transcription (RT)-PCR. *epi10* was found to be highly induced during infection of tomato compared with in vitro grown mycelium (Fig. 2), suggesting that it could play a role in pathogenesis. Altogether, these data suggest that EPI10 is a good candidate to function as a subtilisin inhibitor and to target host proteases similar to EPI1. We therefore proceeded to perform detailed functional characterization of *epi10*.

EPI10 Inhibits the Ser Protease Subtilisin A

To determine whether EPI10 functions as a subtilisin inhibitor as predicted by bioinformatic analyses, we expressed in *Escherichia coli* and affinity purified rEPI10 as a fusion protein with the FLAG epitope tag

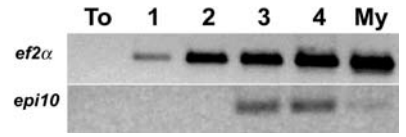

Figure 2. RT-PCR analysis of *epi10* during a time course of colonization of tomato by *P. infestans* and in mycelium. Total RNA isolated from noninfected leaves (To), infected leaves of tomato, 1, 2, 3, or 4 d after inoculation, and from *P. infestans* mycelium grown in synthetic medium (My) was used in RT-PCR amplifications. Amplifications of the constitutive *P. infestans* gene *ef2α* were used as controls to determine the relative expression of *epi10*.

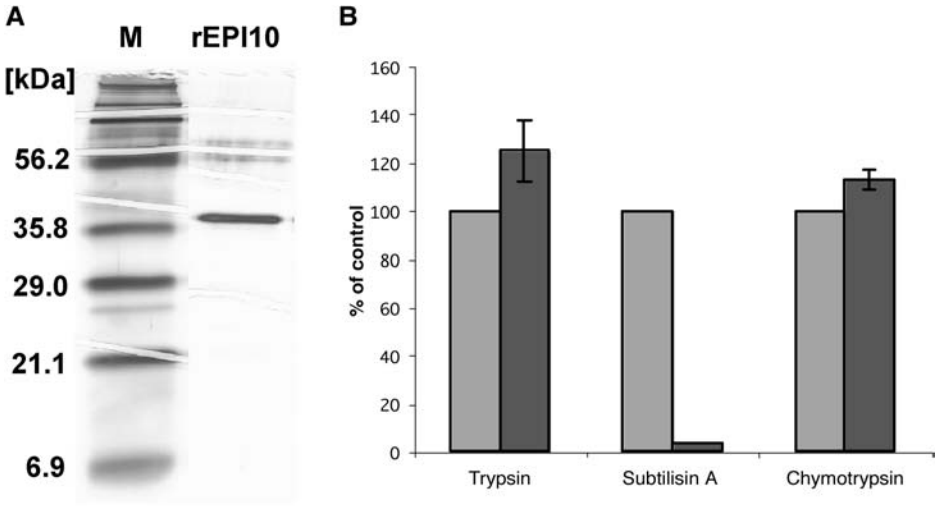

Figure 3. rEPI10 protein inhibits subtilisin A. A, Affinity-purified rEPI10 visualized on SDS-PAGE stained with silver nitrate. Lane M represents M_r markers and lane rEPI10 the purified rEPI10. The numbers on the left represent the molecular masses of the protein standards. B, Protease activities of chymotrypsin, subtilisin A, and trypsin in the absence (gray column) or presence (black column) of rEPI10 were determined using the QuantiCleave Protease Assay kit as described in "Materials and Methods." Activity is expressed as a percentage of total protease activity in the absence of the protease inhibitor. The histograms correspond to the mean of three independent replications of one representative experiment out of three performed. The error bars represent the SES calculated from the three replications.

at the amino terminus. Silver staining of the purified rEPI10 after SDS-PAGE revealed a single band indicating high purity (Fig. 3A). Three major commercial Ser proteases, chymotrypsin, trypsin, and subtilisin A, were selected for inhibition assays with the purified rEPI10. Protease activity was measured with or without rEPI10. In repeated assays, rEPI10 was found to inhibit about 96% of the measured activity of subtilisin A but did not cause apparent inhibition of the other two proteases (Fig. 3B). This result suggests that *epi10* encodes a functional protease inhibitor that specifically targets the subtilisin class of Ser proteases.

EPI10 Interacts with Benzo-(1,2,3)-Thiadiazole-7-Carbothioic Acid *S*-Methyl Ester-Induced Tomato P69 Subtilases

Since the *epi10* gene encodes a functional protease inhibitor of subtilisins and is up-regulated during host infection, we hypothesized that it interacts with tomato proteases, particularly P69 subtilases. To test this hypothesis, we performed coimmunoprecipitation on tomato intercellular fluids incubated with rEPI10 using FLAG antibody covalently linked agarose beads. The eluates were run on SDS-PAGE followed by sequential immunoblotting with P69 and FLAG antisera (Fig. 4). Western-blot analyses with P69 antisera revealed two closely migrated protein bands of approximately 70 kD only from coimmunoprecipitation of samples incubated with rEPI10. This indicates interaction of P69 subtilases with rEPI10. The two closely migrated protein bands reacting with P69 antisera were similar to the ones identified by coimmunoprecipitation of rEPI1 with tomato intercellular fluids (Tian et al., 2004). In both cases, the interacting P69 subtilases were induced by benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) suggesting that the BTH-inducible P69 isoform P69B is likely the main target. Western blot with FLAG antisera confirmed the presence of rEPI10 in the eluted protein complexes. However, rEPI10 was processed in tomato intercellular fluids. Besides the full-length rEPI10, several FLAG-reacting bands smaller than rEPI10 were detected (Fig. 4). It is likely that rEPI10 was processed by BTH-induced tomato proteases given that the

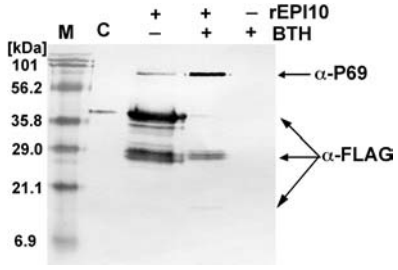

Figure 4. Coimmunoprecipitation of rEPI10 and P69 subtilases using FLAG antisera. Eluates from coimmunoprecipitation of rEPI10 with proteins in tomato intercellular fluids were run on SDS-PAGE gel followed by immunoblotting sequentially with P69 antibody (α-P69) and FLAG antibody (α-FLAG). The bands immunoblotted with each antibody are indicated by arrows. Lane M was loaded with protein M_r standard and the numbers on the left indicate the molecular masses. Lane C was loaded with rEPI10 only. Other lanes were loaded with eluates from coimmunoprecipitation. rEPI10 indicates whether or not rEPI10 was added to the reaction mix. BTH indicates whether or not the intercellular fluids were obtained from plants treated with BTH.

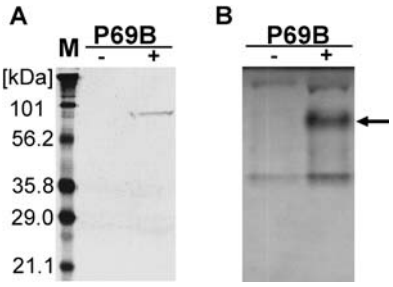

Figure 5. Transient expression of P69B subtilase in *N. benthamiana*. Intercellular fluids were isolated from *N. benthamiana* leaves infiltrated with *A. tumefaciens* containing the binary vector pCB302-3 (−) or pCB-P69B (+). *A. tumefaciens* containing pCB301-P19 was always coinfiltrated in order to enhance protein expression. The intercellular fluids were used in western blot with HA antisera (A) and in-gel protease assay (B). The band corresponding to the protease activity associated with P69B is indicated by an arrow.

extent of degradation of rEPI10 was higher in BTH-treated tomato intercellular fluids compared to water-treated intercellular fluids.

Functional Expression of Tomato P69B Subtilase in *Nicotiana benthamiana*

To test whether rEPI10 inhibits the tomato subtilase P69B and independently confirm the coimmunoprecipitation experiment, we transiently expressed P69B in the apoplast of *N. benthamiana* plants. A construct with P69B fused to epitope tag HA at the C terminus was expressed in *N. benthamiana* plants by agroinfiltration (Kruger et al., 2002). The whole open reading frame of P69B (GenBank accession no. Y17276) encodes a prepropeptide of 745 amino acids that consists of an N-terminal signal peptide of 21 amino acids followed by a 92-amino acid prosequence, and a C-terminal mature protein sequence, which is responsible for the protease activity (Jorda et al., 1999). We extracted intercellular fluids from infiltrated leaves of *N. benthamiana* and performed western blot with HA antisera to detect P69B-HA. A distinct band of approximately 70 kD was detected in intercellular fluids from leaves infiltrated with *Agrobacterium tumefaciens* carrying the plasmid pCB-P69B, but not from leaves infiltrated with *A. tumefaciens* containing the empty binary vector, indicating that the mature form of P69B-HA was successfully expressed (Fig. 5A). To test if the expressed fusion protein P69B-HA is a functional protease, 10 μL intercellular fluids from two treatments were used in in-gel protease assay. An additional protease band was clearly visible in the P69B-expressing sample but not in the control and is likely to reflect the protease activity of P69B (Fig. 5B).

EPI10 Inhibits the Tomato PR Ser Protease P69B

To test whether rEPI10 inhibits the protease activity of P69B, *N. benthamiana* intercellular fluids containing the P69B-HA protein were incubated with or without rEPI10 and the protease activity was detected by in-gel protease assay. rEPI10 completely inhibited the protease activity of P69B and also reduced the activity of two other protease bands from *N. benthamiana* (Fig. 6). These bands are likely to be subtilisin-like Ser proteases of *N. benthamiana*, perhaps homologs of tomato P69 subtilases. However, their exact identity remains unknown. This experiment independently confirms that EPI10 inhibits P69 subtilases and that it specifically targets P69B.

DISCUSSION

Plant apoplastic proteases have long been tied to defense responses (Tornero et al., 1997; Jorda et al., 1999; van Loon and van Strien, 1999; Kruger et al., 2002; Xia et al., 2004). Previously, EPI1, a two-domain Kazal-like protease inhibitor from *P. infestans*, was determined to inhibit and interact with the PR P69B subtilisin-like Ser protease of the host plant tomato, suggesting that inhibition of plant defense-related proteases is a virulence mechanism that *P. infestans* employs to suppress plant defense. In this study, a second Kazal-like extracellular protease inhibitor of *P. infestans*, the three-domain EPI10, was also found to inhibit and interact with P69B. The *epi10* gene is significantly up-regulated during colonization of tomato. Based on its biological activity and expression pattern, EPI10 may function as a disease effector molecule similar to EPI1.

The finding that *P. infestans* evolved two distinct protease inhibitors to target the same plant protease suggests that inhibition of P69B could be an important infection mechanism for *P. infestans*. The EPI1 and EPI10 proteins are divergent in amino acid sequence, molecular mass, and number of Kazal domains, but the expression patterns of *epi1* and *epi10* during infection of tomato by *P. infestans* is similar (Tian et al.,

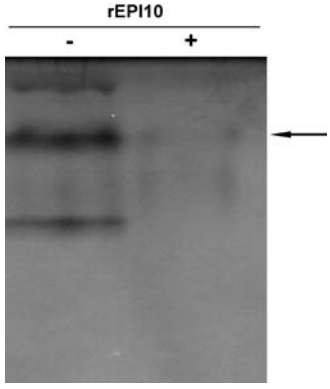

Figure 6. EPI10 inhibits P69B subtilase. Intercellular fluids isolated from *N. benthamiana* leaves coinfiltrated with *A. tumefaciens* containing pCB-P69B and *A. tumefaciens* containing pCB301-P19 were incubated in the absence (−) or presence of rEPI10 (+) and the remaining protease activity was analyzed using zymogen in-gel protease assays. The arrow indicates the band corresponding to the protease activity associated with P69B.

2004). Both genes are up-regulated, although the induction level of *epi10* is higher than *epi1*. The concurrent expression pattern of these two genes suggests that they could play complementary roles in order to completely inhibit P69B, a highly abundant apoplastic protein.

The Laskowski algorithm predicted that the first domain of EPI1 (EPI1a) and the second domain of EPI10 (EPI10b) are strong subtilisin inhibitors (K_i lower than 10^{-9} M). Our experimental data indeed confirmed that EPI10 is an inhibitor of subtilisn A that also inhibits and interacts with P69B subtisin-like Ser protease of the host plant tomato. Moreover, the EPI1 prediction is also consistent with experimentally determined data (Tian et al., 2004). In parallel experiments, we purified and assayed four additional recombinant EPI proteins, EPI2, EPI3, EPI5, and EPI12, of *P. infestans* (M. Tian and S. Kamoun, unpublished data). None of these proteins exhibited inhibition activity against subtilisin A as predicted by the algorithm. The Laskowski additivity-based sequence to reactivity algorithm is, therefore, a useful tool to help identify the right candidate protein prior to embarking in biochemical analyses. Future inhibition assays with individual Kazal domains of EPI1 and EPI10 will further assess the applicability of the algorithm to the study of *P. infestans* Kazal domains.

The EPI1a and EPI10b domains that were predicted to inhibit subtilisin A are atypical Kazal domains that lack Cys 3, Cys 6, and the corresponding disulfide bridge (Fig. 2B). These atypical two disulfide bridge domains are common in plant pathogenic oomycetes. Among the 56 Kazal domains identified from five plant pathogenic oomycetes, 14 belong to this class (Tian et al., 2004). These 14 domains occur in 14 different proteins from three Phytophthora species, *P. infestans*, *P. ramorum*, and *P. sojae*. Interestingly, a phylogenetic analysis of all 56 oomycete Kazal domains revealed that these 14 atypical domains are monophyletic, suggesting that the loss of the Cys 3-Cys 6 disulfide bridge in Phytophthora domains predates speciation (M. Tian and S. Kamoun, unpublished data). Further experiments to characterize these atypical two disulfide bridge domains would shed some light on Kazal domain structure and help understand the biological functions of the diverse Kazal-like inhibitors of plant pathogenic oomycetes.

In coimmunoprecipitation of rEPI10 with tomato intercellular fluids, we found that some proteases proteolytically processed this protein. Independent experiments performed by incubating rEPI10 in water-treated and BTH-induced tomato intercellular fluids followed by immunoblotting with FLAG antisera confirmed that EPI10 is particularly unstable in BTH-induced tomato fluids (data not shown). However, despite rEPI10 processing, we still pulled down P69 subtilases in the coimmunoprecipitation experiment. Several explanations could account for this result. First, processed amino-terminal fragments of rEPI10 containing one or two Kazal domains might be functional in interacting with P69. Indeed, there are many examples of active shorter peptides derived from a multidomain inhibitor and some Kazal inhibitors are naturally processed into single-domain peptides (Scott et al., 1987; Magert et al., 1999; Mitsudo et al., 2003; Jayakumar et al., 2004; Mende et al., 2004). Second, rEPI10 was not totally processed, and some intact molecules could still be detected in the eluates (Fig. 4). These remaining full-length molecules might account for the interaction with P69. Further experiments to characterize the nature and activity of the processed EPI10 fragments will clarify this issue.

In this study, we were successful in expressing a functional tomato P69B in *N. benthamiana* using *A. tumefaciens*-mediated transient expression (agroinfiltration). Such an in planta expression system provides an ideal tool for functional characterization of plant proteases. A large number of plant proteases have a prodomain that must be removed for the protease to become active. Commonly used heterologous expression systems, such as *E. coli* expression, are usually not effective for proteases (Bromme et al., 2004). During the expression of proteases in *E. coli*, the presence of prodomains is essential for correct folding. Therefore, additional steps to remove the prodomains are required to obtain active enzymes (Bromme et al., 2004). Heterologous expression of proteases in planta eliminates such steps. In *N. benthamiana* plants, the prodomains of plant proteases are expected to be processed and active proteases can be obtained in vivo. The tomato Cys protease Rcr3 was successfully expressed in *N. benthamiana* using agroinfiltration (Kruger et al., 2002). In this study, tomato P69B subtilase was also effectively expressed by agroinfiltration resulting in a functional protein. With the availability of genome sequence from an increased number of plant species, a large number of plant proteases have been discovered. For example, the Arabidopsis genome has over 550 protease sequences representing all five catalytic types: Ser, Cys, Asp, metallo, and Thr (Beers et al., 2004). The availability of efficient protease expression systems will be critical for characterizing the functions of these plant proteases.

MATERIALS AND METHODS

Prediction of Inhibition Constants

The fourteen *Phytophthora infestans* Kazal-like extracellular Ser protease inhibitors (EPI1–EPI14) that contain a total of 21 Kazal domains were described previously (Tian et al., 2004). The putative 10 hypervariable contact residues of each domain were identified based on similarity to canonical animal Kazal domains (Read et al., 1983; Laskowski et al., 1987; Lu et al., 1997). Predicted inhibition constants of *P. infestans* Kazal domains against the Ser protease subtilisin A (Carlsberg) were generated by Drs. M.A. Qasim and M. Laskowski Jr. (Purdue University, West Lafayette, IN) with the additivity-based sequence to reactivity algorithm (Laskowski algorithm; Lu et al., 2001).

Phytophthora Strain and Culture Conditions

P. infestans isolate 90128 (A2 mating type, race 1.3.4.7.8.9.10.11) was routinely grown on rye agar medium supplemented with 2% Suc (Caten

and Jinks, 1968). For RNA extraction, plugs of mycelium were transferred to modified Plich medium (Kamoun et al., 1993) and grown for 2 weeks before harvesting.

Plant Growth, BTH Treatment, and Infection by *P. infestans*

Tomato (*Lycopersicon esculentum*) cultivar Ohio 7814 and *Nicotiana benthamiana* plants were grown in pots at 25°C, 60% humidity, under 16-h-light/8-h-dark cycle. We used the SA analog BTH to induce PR proteins. BTH treatment of tomato plants followed the exact same procedure described previously (Tian et al., 2004). Time courses of *P. infestans* infection of tomato leaves were performed as described earlier (Kamoun et al., 1998; Tian et al., 2004).

Bacterial Strains and Plasmids

Escherichia coli XL1-Blue and *Agrobacterium tumefaciens* GV3101 were used in this study and were routinely grown in Luria-Bertani media (Sambrook et al., 1989) at 37°C and 28°C, respectively. Plasmid pFLAG-EPI10 was constructed by cloning PCR-amplified DNA fragment corresponding to the mature sequence of EPI10 (GenBank accession no. AY586282) into the *Eco*RI and *Kpn*I sites of pFLAG-ATS (Sigma, St. Louis), a vector that allows secreted expression in *E. coli*. The oligonucleotides EPI10-F1 (5′-GCGGAATTCTGCAGACGACAACTGCTCTTTTGG-3′) and EPI10-R1 (5′-GCGGGTACCTTATGTGGCTACCATCTCGTTAC-3′) were used to amplify the fragment. The introduced *Eco*RI and *Kpn*I restriction sites are underlined. The N-terminal sequence of the processed rEPI10 protein is "DYKDDDDKVKLLENS**ADDNCSFGCL**…" The FLAG epitope sequence is underlined, and the first 10 amino acids of mature EPI10 are shown in bold. Plasmid pCB-P69B was constructed by cloning a PCR-amplified DNA fragment corresponding to the open reading frame of the *P69B* gene (GenBank accession no. Y17276) fused with the HA tag (YPYDVPDYA) at the C terminus into the *Bam*HI and *Spe*I sites of the binary vector pCB302-3 (Xiang et al., 1999). The oligonucleotides P69B-F1 (5′-GCGGGATCCATGGGATTATTGAAAATCCTTCTTGTTTTC-3′) and P69B-R1 (5′-GCGTCTAGACTAagcgtaatctggaacatcgtatgggtaGGCAGACACAACTGC AATTGGAC-3′) were used to amplify the *P69B* fragment. The introduced *Bam*HI and *Xba*I sites are underlined. The introduced HA-encoding sequence is shown in lowercase.

Semiquantitative RT-PCR

RNA isolation and semiquantitative RT-PCR were performed as described earlier (Torto et al., 2003; Tian et al., 2004). The oligonucleotides EPI10-F1 and EPI10-R1, designed for cloning *epi10* into pFLAG-ATS vector, were used to detect *epi10* transcripts by RT-PCR. The expression of *epi10* was controlled with the constitutively expressed *P. infestans* elongation factor 2 alpha (*ef2α*) gene using the primer pair described previously (Torto et al., 2002).

SDS-PAGE and Western-Blot Analyses

Proteins were subjected to 10% to 15% SDS-PAGE as previously described (Sambrook et al., 1989). Following electrophoresis, gels were stained with silver nitrate following the method of Merril et al. (1981), or the proteins were transferred to supported nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) using a Mini Trans-Blot apparatus (Bio-Rad Laboratories). Detection of antigen-antibody complexes was carried out with a western-blot alkaline phosphatase kit (Bio-Rad Laboratories). Antisera to P69 subtilases were raised against a peptide specific for the tomato P69 family (Tian et al., 2004). Monoclonal anti-FLAG M2 and anti-HA antibodies were purchased from Sigma.

Expression and Purification of rEPI10

Expression and purification of rEPI10 was conducted as described previously for other pFLAG-ATS derived constructs (Kamoun et al., 1997; Tian et al., 2004). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories). To determine the purity, 0.5 μg of the purified protein was run on an SDS-PAGE gel followed by staining with silver nitrate.

Transient Expression of P69B Subtilase in Planta

Transient expression of P69B-HA in planta was performed according to the agroinfiltration method described previously (Kruger et al., 2002). *A. tumefaciens* strains carrying plasmids pCB-P69B, empty vector pCB302-3 (Xiang et al., 1999), and pCB301-P19 (Win and Kamoun, 2004) were used. pCB301-P19 is a construct expressing the P19 protein of tomato bushy stunt virus, a suppressor of posttranscriptional gene silencing in *N. benthamiana* (Voinnet et al., 2003). Overnight agrobacteria cultures were harvested by centrifugation at 2,000 g for 20 min, and resuspended in 10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 150 μM acetosyringone. Resuspended agrobacteria cultures of pCB-P69B or pCB302-3 with an optical density (OD_{600}) of 1.0 were mixed with equal volumes of a culture of pCB301-P19 with an optical density (OD_{600}) of 2.0. The mixtures were kept at room temperature for 3 h and then infiltrated into leaves of 6-week-old *N. benthamiana* plants. Intercellular fluids from infiltrated leaves were isolated 5 d after infiltration.

Isolation of Intercellular Fluids

Intercellular fluids were prepared from tomato and *N. bethamiana* leaves according to the method of de Wit and Spikman (1982). For tomato leaves, a 0.24-M sorbitol solution was used as extraction buffer. For leaves from *N. benthamiana*, a solution of 300 mM NaCl, 50 mM $NaPO_4$, pH 7 (Kruger et al., 2002) was used as extraction buffer. The intercellular fluids were filter sterilized (0.45 μM) and were used immediately or stored at −20°C.

In-Gel Protease Assays

In-gel protease assays with intercellular fluids from *N. benthamiana* were performed using Bio-Rad's zymogram buffer system as described previously (Tian et al., 2004).

Assays of Protease Inhibition

Inhibition assays of commercial Ser proteases by rEPI10 was performed by the colorimetric QuantiCleave Protease Assay kit (Pierce, Rockford, IL) as described previously (Tian et al., 2004). A total of 20 pmol of rEPI10 was preincubated with 20 pmol of trypsin (Pierce), chymotrypsin (Sigma), or subtilisin A (Carlsberg; Sigma) in a volume of 50 μL for 30 min at 25°C, and followed by incubation with 100 μL of succinylated casein (2 mg/mL) in 50 mM Tris buffer, pH 8, at room temperature for 20 min. Protease activity was measured as A_{405} using HTS 7000 Bio Assay Reader (Perkin-Elmer Applied Biosystems, Foster City, CA) 20 min after the addition of chromogenic reagent 2,4,6-trinitrobenzene sulfonic acid, which reacts with the primary amine of digested peptide and produces the color reaction that can be quantified by the absorbance reader.

Inhibition assays of plant protease P69B by rEPI10 were carried out with in-gel protease assays. A total of 20 pmol of rEPI10 were preincubated with 10 μL of intercellular fluids for 30 min at 25°C and the remaining protease activity was detected.

Coimmunoprecipitation

Coimmunoprecipitation of rEPI10 and tomato intercellular fluids was performed using the FLAG-tagged protein immunoprecipitation kit (Sigma) as described previously (Tian et al., 2004). A total of 800 pmol of purified rEPI10 were preincubated with 200 μL of tomato intercellular fluids for 20 min at 25°C. Forty microliters of anti-FLAG M2 resin was added and incubated at 4°C for 2 h with gentle shaking. The precipitated protein complexes were eluted in 60 μL of FLAG peptide solution (150 ng/μL) and were analyzed by SDS-PAGE and western-blot analyses.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY586282.

ACKNOWLEDGMENTS

We are grateful to M.A. Qasim and Michael Laskowski Jr. from Purdue University for predicting the inhibition constants and sharing their knowledge of Kazal inhibitors. We also thank Diane Kinney from Ohio State University for technical assistance.

Received February 13, 2005; revised April 22, 2005; accepted April 22, 2005; published June 24, 2005.

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