A Kazal-like Extracellular Serine Protease Inhibitor from 
*Phytophthora infestans* Targets the Tomato Pathogenesis-related 
Protease P69B*

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The oomycetes form one of several lineages within the 
eukaryotes that independently evolved a parasitic lifestyle 
and consequently are thought to have developed 
adversative mechanisms of pathogenicity. The oomycete 
*Phytophthora infestans* causes late blight, a ravaging 
disease of potato and tomato. Little is known about pro-
cesses associated with *P. infestans* pathogenesis, partic-
ularly the suppression of host defense responses (4–7). 
We describe and functionally characterize an extracellular 
protease inhibitor, EPI1, from *P. infestans*. EPI1 con-
tains two domains with significant similarity to the 
Kazal family of serine protease inhibitors. Database 
ssearches suggested that Kazal-like proteins are mainly 
restricted to animals and apicomplexan parasites but 
appear to be widespread and diverse in the oomycetes. 
Recombinant EPI1 specifically inhibited subtilisin A 
among major serine proteases and inhibited and inter-
acted with the pathogenesis-related P69B subtilisin-like 
serrine protease of tomato in intercellular fluids. The 
ep1 and P69B genes were coordinately expressed and 
up-regulated during infection of tomato by *P. infestans*. 
Inhibition of tomato proteases by EPI1 could form a 
novel type of defense-counterdefense mechanism 
be tween plants and microbial pathogens. In addition, this 
study points to a common virulence strategy between 
the oomycete plant pathogen *P. infestans* and several 
mmalian parasites, such as the apicomplexan 
*Toxoplasma gondii*.

Parasitic and pathogenic lifestyles have evolved repeatedly 
in eukaryotes (1). Several parasitic eukaryotes represent deep 
phylogenetic lineages, suggesting that they feature unique mo-
elar processes for infecting their hosts. One such lineage is 
formed by 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 (1–3). One of the most no-
torious and destructive oomycete is the Irish famine pathogen, 
*Phytophthora infestans*. This species causes late blight, a re-
emerging and ravaging disease of potato and tomato (4–7). 
During the early stages of infection, *P. infestans* requires living 
host cells but later causes extensive necrosis of host tissue, a 
lifestyle that is known as hemibiotrophy. As with other biotro-
phic plant pathogens, processes associated with *P. infestans* 
pathogenesis are thought to include the suppression of host 
defense responses (3, 8, 9). In *P. infestans*, water-soluble glu-
cans have been reported to suppress host defenses in a plant 
cultivar-specific manner (10–12). Nevertheless, the molecular 
basis of suppression of host defenses by *Phytophthora* remains 
poorly understood (3). It is tempting to speculate that unique 
classes of suppressor genes have been recruited to aid in infec-
tion and counteract host defenses during the evolution of 
pathogenesis in the oomycete lineage.

Parasitic eukaryotes often face inhospitable environments in 
their hosts. For example, parasites that colonize or transit 
through the mammalian digestive tract must adapt to the 
diverse and abundant array of proteases secreted in the gastric 
juices (13–15). Some of these parasites secrete inhibitors that 
target host proteases and may aid in survival and colonization 
of the host. For instance, the apicomplexan obligate parasite 
*Toxoplasma gondii* secretes TgPI-1 and TgPI-2, four-domain 
serrine protease inhibitors of the Kazal family (15–19), and the 
testinal hookworm *Ancephalostoma ceylanicum* secretes an 
8-kDa broad spectrum serrine protease inhibitor of the Kunitz 
family (14). In plants, the apoplast (intercellular fluid) forms a 
protease-rich environment that is colonized by many patho-
gens, including *P. infestans* and the fungus *Cladosporium ful-

In tomato, apoplastic proteases are integral components of 
the plant defense response. Serine proteases of the P69 
brasilase family have long been tied to pathogen defense, and 
two isoforms, P69B and P69C, are known as pathogenesis-
related proteins (PR-7 class) (20–22). More recently, an apo-
mic-like protease inhibitor, Rcr3, was shown to be 
required for specific resistance to *C. fulvum* (23). In addition, 
several *C. fulvum* extracellular proteins are processed or 
degraded by host proteases in the apoplast, resulting in altered 
functionality (24, 25).

Despite the importance of extracellular proteases in plant 
defense, to date no protease inhibitor has been reported from 
microbial plant pathogens. In this paper, we describe and func-
tionally characterize an extracellular protease inhibitor, EPI1, 
from *P. infestans*. EPI1 contains two domains with significant 
similarity to the Kazal family of serine protease inhibitors, 
which also occurs in many animal species and in apicomplexan 
parasites. *In vitro* studies indicated that recombinant EPI1 
(rEPI1) specifically inhibited subtilisin A among the major 
serrine proteases. rEPI1 was further demonstrated to inhibit 
and interact with tomato P69B subtilisin-like serrine protease.

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*The abbreviations used are: rEPI1, recombinant EPI1; BTH, benzo-
(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester; EST, expressed 
sequence tag; RT, reverse transcriptase.*

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The ep11 and P69B genes were coordinately expressed and up-regulated during infection of tomato by *P. infestans*. Overall these results suggest that inhibition of tomato proteases by *P. infestans* EP11 could form a novel type of defense-counterdefense mechanism between plants and microbial pathogens. In addition, this study points to a common virulence strategy among malarial parasites, such as the apicomplexan *T. gondii*.

**MATERIALS AND METHODS**

**Phytophthora Strains and Culture Conditions**— *P. infestans* isolate 90126 (A2 mating type, race 1.3.7.8.9.10.11) was used throughout the study. *P. infestans* 90128 was routinely grown on rye agar medium supplemented with 2% sucrose (28). For RNA extraction, plucks of mycelium were transferred to modified Pich medium (27) and grown for 2–3 weeks before harvesting.

**Bacterial Strains and Plasmids**— *Escherichia coli* XLI-Blue was used in this study and was routinely grown at 37 °C in LB medium (28). Plasmid pFLAG-EP11 was constructed by cloning PCR-amplified DNA fragment corresponding to the mature sequence of EP11 into the HindIII site of pFLAG-ATS (Sigma), a vector that allows secreted expression in *E. coli*. The oligonucleotides EP11-F1 (5'-GCCGGAGTCCTCGA- GCGGATGTCCGTTACG-3') and EP11-R1 (5'-CCGGCACTCCTCCCTGTT-3') were used to amplify the fragment. The introduced HindIII restriction sites are underlined. The N-terminus of the processed FLAG-rEP11 protein is DYKDEDDDKVKL.

**Sequence Analyses**—GC counting was performed as described elsewhere (31). PeaFinder and signal peptide predictions were performed as described by Torto et al. (32). Similarity searches were performed locally on an Intel Linux or a Mac OSX work station or through the internet on the NCGR (www.ncgr.org) and Whitehead Institute web servers (www-genome.wi.mit.edu/resources.html). Search programs included BLAST (33), and the similarity search programs implemented in the BLOCKS (34), pfam (35), SMART (36), and InterPro (37) websites. The examined sequence databases included GenBank™ nonredundant, the BLOCKS (34), pfam (35), SMART (36), and InterPro (37) websites. The examined sequence databases included GenBank™ nonredundant, Databases (www-genome.wi.mit.edu/resources.html). Multiple alignments were done using the program CLUSTAL-X (40). The *P. infestans* and *Phythophthora brassicae* sequences described in this paper were deposited in GenBank™ under accession numbers M856723-9, M856793-1, and M8568126-8, respectively. Other sequences were obtained from the NCBI nr, dBEST, or Trace Archive data bases (www.ncbi.nlm.nih.gov) (Table I).

**RNA Isolation, Northern Blot, and RT-PCR Analyses**—RNA isolation and Northern blot hybridizations were performed as described earlier (32). Probes for *ep11*, *actA*, and *tomato α-tubulin* were generated by random primer labeling using gel-purified fragments digested or PCR products from the corresponding cDNA clones (Ref. 41 for this study). The probe for tomato P69B was generated from a gel-purified RT-PCR fragment amplified from total RNA isolated from infected tomato tissue. For RT-PCR, total RNA was treated with DNA-free™ (Ambion, Austin, TX) to remove contaminating DNA, and first-strand cDNAs were synthesized using the ThermoScript™ RT-PCR system from 5 μg of total RNA following the instructions of the manufacturer (Invitrogen). PCR amplifications were carried out with 0.005% of the cDNA product. The oligonucleotide primer pairs, P69B-RTR1 (5'-TGGCAGG- GTCGGAGTTCCGAGGG-3') and P69B-RTR1 (5'-CATTGGATCACAACAAAATGCAATTG-3'), P69B-RTR1 (5'-CATGACCTGGCGCTGGATGTT-3') and P69A-RTR1 (5'-CATTGGATCACAACAAAATGCAATTG-3'), were designed to be gene-specific based on the published *P. infestans* gene sequences (21) and were used for the amplification of *P69A*, *P69B*, and *P69D* sequences, respectively. The oligonucleotides EP11-F1 and EP11-R1, previously used for cloning *ep11* into pFLAG-ATS vector, were used to detect *ep11* transcripts by RT-PCR. Primer specificity was confirmed by sequencing the RT-PCR products. The expression of *P69A*, *P69B*, and *P69D* was controlled with primer pair EP1e-F1 (5'-GGCTGCTGTAACACGATTGCGCTTCG-3') and EP1e-R1 (5'-CCGACATCATCGTCAGATCTCGACT-3'), which are specific for the constitutively expressed tomato elongation factor 1a gene (42). The expression of *ep11* was controlled with *P. infestans* elongation factor 2a gene using the primer pair described previously (43).

**SDS-PAGE and Western Blot Analyses**—Proteins were subjected to 10–15% SDS-PAGE as previously described (28). Following electrophoresis, the gels were stained with silver nitrate following the method of Merril et al. (44) or stained with Coomassie Brilliant Blue (28), or the proteins were transferred to supported nitrocellulose membranes (Bio-Rad) using a Mini Trans-Blot apparatus (Bio-Rad). Detection of antigen-antibody complexes was carried out with a Western blot alkaline phosphatase kit (Bio-Rad). Antisera to P69 subtilases were produced by immunizing rabbits with the keyhole limpet hemocyanin-conjugated peptide, H2N-TTHTPSFLGLQQNC-amide. The sequence underlined is located at the N terminus of mature P69B and P69D and was chosen for its highly antigenic characteristics and conservation among *P. infestans* proteins. Selection of peptides for highly antigenic characteristics, peptide synthesis, and conjugation, as well as antisera production, was performed by Rockland Immunochemicals (Gilbertsville, PA). In Western blot analyses, the antisera to the P69 peptide reacted only with ~70-kDa bands from tomato intercellular fluids.

**Expression and Purification of rEP11**—Expression of rEP11 from pFLAG-EP11 was conducted as described previously (45). Cultures of *E. coli* XLI-blue containing pFLAG-EP11 were diluted (1:100) in LB medium containing ampicillin (50 μg/ml) and incubated at 37 °C. When the *A*₆₀₀ of the cultures reached 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. The cultures were further incubated for 5–6 h before processing. rEP11 was recovered from the culture supernatant and was purified by immuno-affinity using gravity column packed with anti-FLAG M2 affinity gel (Sigma). The proteins were eluted with 0.1 M glycine, pH 3.5, and immediately equilibrated to neutral pH with 20 μl of 1 M Tris, pH 8.0, for each 1-ml eluted fraction. The protein concentrations were determined using the Bio-Rad protein assay. To determine the purity of rEP11, 0.5 μg of the purified protein was run on a SDS-PAGE gel followed by staining with silver nitrate.

**Assays of Protease Inhibition**—Inhibition assays of commercial serine proteases by rEP11 were performed by the colorimetric Quantimet (44) or stained with Coomassie Brilliant Blue (28), or the proteins were transferred to supported nitrocellulose membranes (Bio-Rad) using a Mini Trans-Blot apparatus (Bio-Rad). Detection of antigen-antibody complexes was carried out with a Western blot alkaline phosphatase kit (Bio-Rad). Antisera to P69 subtilases were produced by immunizing rabbits with the keyhole limpet hemocyanin-conjugated peptide, H2N-TTHTPSFLGLQQNC-amide. The sequence underlined is located at the N terminus of mature P69B and P69D and was chosen for its highly antigenic characteristics and conservation among *P. infestans* proteins. Selection of peptides for highly antigenic characteristics, peptide synthesis, and conjugation, as well as antisera production, was performed by Rockland Immunochemicals (Gilbertsville, PA). In Western blot analyses, the antisera to the P69 peptide reacted only with ~70-kDa bands from tomato intercellular fluids.

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Protease Inhibitors from Phytophthora

**TABLE I**

| Species                | Protein | GenBank™ accession number | Signal peptide | Expression stage | Number of Kazal-like domains | PI residue |
|------------------------|---------|---------------------------|----------------|------------------|------------------------------|------------|
| *P. infestans*         | EP1     | AY586273                  | Yes            | Infected tomato  | 2                            | Asp, Asp   |
|                        | EP2     | AY586274                  | Yes            | Mycelium, H₂O₂-treated | 2                            | Asp, Asp   |
|                        | EP3     | AY586275                  | Yes            | Genomic sequence | 1                            |            |
|                        | EP4     | AY586276                  | Yes            | Mycelium, nitrogen starvation | 3                            | Thr, Thr, Asp, Asp |
|                        | EP5     | AY586277                  | Yes            | Mating culture   | 1                            | Arg        |
| *P. infestans*         | EP6     | AY586278                  | NA             | Infected tomato  | 2                            | Asp, Asp   |
|                        | EP7     | AY586279                  | Yes            | Genomic sequence | 1                            | Asp        |
|                        | EP8     | AY586280                  | Yes            | Genomic sequence | 1                            | Asp        |
|                        | EP9     | AY586281                  | Yes            | Mycelium, non-sporulating growth | 1                            | Asp, Asp   |
|                        | EP10    | AY586282                  | Yes            | Zoospores        | 3                            | Asp, Asp   |
| *P. infestans*         | EP11    | AY586283                  | Yes            | Mating culture   | 1                            | Asp        |
|                        | EP12    | AY586284                  | Yes            | Infected potato, germinating cysts | 1                            | Ser        |
| *P. infestans*         | EP13    | 178869887                 | Yes            | Genomic sequence | 1                            | Glu        |
|                        | EP14    | 178923959                 | Yes            | Genomic sequence | 1                            | His        |
| *P. sojae*             | PsoEP1  | CF842223                  | Yes            | Infected soybean  | 4                            | Ala, Glu, Lys, Ala |
| *P. sojae*             | PsoEP2  | AA024652                  | Yes            | Mycelium        | 1                            |            |
| *P. sojae*             | PsoEP3  | 274204995                 | Yes            | Genomic sequence | 3                            | Met, Asp, Glu |
| *P. sojae*             | PsoEP4  | 27352724                  | Yes            | Genomic sequence | 3                            | Asp, Thr, Asp |
| *P. sojae*             | PsoEP5  | 273752552                 | Yes            | Genomic sequence | 1                            | Arg        |
| *P. sojae*             | PsoEP6  | 273759065                 | Yes            | Genomic sequence | 1                            | Glu        |
| *P. sojae*             | PsoEP7  | 234111439                 | Yes            | Genomic sequence | 1                            | Asp        |
| *P. sojae*             | PsoEP8  | 273566013                 | Yes            | Genomic sequence | 1                            | Asp        |
| *P. sojae*             | PsoEP9  | 274071208                 | Yes            | Genomic sequence | 1                            | Arg        |
| *P. sojae*             | PsoEP10 | 273704880                 | Yes            | Genomic sequence | 1                            | Ala        |
| *P. sojae*             | PsoEP11 | 324096913                 | Yes            | Genomic sequence | 1                            |            |
| *P. sojae*             | PsoEP12 | 324106054                 | Yes            | Genomic sequence | 1                            | Asp        |
| *P. ramorum*           | PramEP1 | 303599335                 | Yes            | Genomic sequence | 3                            | Asp, Met, Glu |
| *P. ramorum*           | PramEP4 | 304926165                 | Yes            | Genomic sequence | 3                            | Asp, Thr, Asp |
| *P. ramorum*           | PramEP5 | 304247992                 | Yes            | Genomic sequence | 1                            | Arg        |
| *P. ramorum*           | PramEP9 | 303791515                 | Yes            | Genomic sequence | 1                            | Arg        |
| *P. ramorum*           | PramEP10| 303477516                 | Yes            | Genomic sequence | 3                            | Asp, Asp   |
| *P. ramorum*           | PramEP11| 30378321                  | Yes            | Genomic sequence | 1                            |            |
| *P. brassicaceae*      | PbrEP1  | AY588906                  | Yes            | Mycelium, nitrogen starvation | 2                            | Asn, Met   |
| *P. brassicaceae*      | PbrEP2  | AY589087                  | NA             | Mycelium        | 1                            | His        |
| *P. halstedii*         | PhaEP1  | CB174657                  | Yes            | Infected sunflower | 1                            | Arg        |

NA, not available.

Ti (Trace Identifier) number from NCBI Trace Archive (www.ncbi.nlm.nih.gov/Traces/trace.cgi).

SignalP (48) analysis of the predicted protein identified a 16-26372

65% sequence identity to rEP1 from *P. brassicaceae*, a 24% sequence identity to rEP1 from *P. sojae*, and a 21% sequence identity to rEP1 from *P. ramorum*. The alignment results are consistent with the phylogenetic analysis, suggesting that Kazal-like protease inhibitors are highly conserved among oomycetes and have been diversely distributed across the lineages of oomycetes. The predicted amino acid sequences of Kazal-like protease inhibitors from various oomycete species were used to design specific antibodies for future experiments, such as ELISA or western blotting, to further verify the presence and expression of these inhibitors in different oomycete species.

time. Initial reaction velocities were measured by monitoring the absorbance change at 405 nm over reaction time using the HTS 7000 Bio Assay Reader (PerkinElmer Life Sciences). K<sub>app</sub> was determined following the method described by Morris et al. (15). The slope of the linear plot of [V<sub>i</sub>/V<sub>i</sub> - 1 versus 1/I<sub>i</sub>] was estimated as 1/K<sub>app</sub>, K<sub>app</sub> was converted to K<sub>i</sub> according to the formula K<sub>i</sub> = K<sub>app</sub>(1 + [S]/K<sub>max</sub>) (46). Varying concentrations of substrates were incubated with 2 pmol of subtilisin A in a total volume of 200 μl under the conditions described above, and the initial velocities were measured by monitoring the absorbance at 405 nm. The K<sub>i</sub> was determined graphically by double-reciprocal Lineweaver-Burk plots of 1/v<sub>i</sub> versus 1/s. 

Inhibition assays of plant proteases by rEP1 were carried out with the QuantiCleave™ Protease Assay Kit (Pierce) and in-gel protease assays using the Bio-Rad zymogram buffer system. For the first method, 50 μl of intercellular fluids were preincubated with or without 10 pmol of rEP1 at 25 °C for 30 min, and the protease activities were subsequently measured. For the in-gel protease assays, 10 pmol of rEP1 were preincubated with 8 μl of intercellular fluids for 30 min at 25 °C and then mixed with zymogram sample buffer and loaded on a 10% SDS-polyacrylamide gel without boiling or addition of reducing reagents. Following electrophoresis, the gel was incubated in 1× zymogram renaturation buffer for 30 min. Then the gel was incubated in 1× zymogram development buffer for 4 h at 37 °C before staining with 0.5% Coomassie Brilliant Blue.

**RESULTS**

**EP1 Belongs to the Kazal Family of Protease Inhibitors**—We mined an EST data set generated from tomato leaves 3 days after infection with *P. infestans* using two methods: 1) GC counting to distinguish between Phytophthora and tomato sequences (31) and 2) PexFinder to identify cDNAs encoding extracellular proteins (32). 488 of 2808 ESTs examined showed a GC content higher than 53%. Of these 42 were predicted to encode extracellular proteins using the criteria of Torto et al. (32). These ESTs were then annotated by similarity and motif searches against public databases. One EST, P0C064G6 (GC content, 57.4%), showed similarity to proteins of the Kazal serine protease inhibitor family. DNA sequencing of the full cDNA revealed an open reading frame of 450 bp corresponding to a predicted translated product of 149 amino acids (Fig. 1A). SignalP (48) analysis of the predicted protein identified a 16-

**Tandem Mass Spectrometric Sequencing**—Tandem mass spectrometric sequencing was performed at the proteomics facility of The Cleveland Clinic Foundation (Cleveland, OH). The selected protein band was cored from the gel, and protein digestion was carried out as previously described (47). The liquid chromatography-mass spectrometry system used is a Finnigan LCQ-Deca ion trap mass spectrometer system with a Protona microelectrospray ion source interfaced to a self-packed 10 cm × 75 μm Phenomenex Jupiter C18 reversed-phase capillary chromatography column. 2-μl volumes of the peptide extract were injected, and the peptides were eluted from the column by an acetonitrile, 0.05 M acetic acid gradient at a flow rate of 0.2 μl/min. The microelectrospray ion source was operated at 2.5 kV. The digest was analyzed using the data-dependent multitask capability of the instrument resulting in ~1000 collision-induced dissociation spectra of ions ranging in abundance over several orders of magnitude. The data were analyzed by using all collision-induced dissociation spectra collected in the experiment to search the NCBI nonredundant data base with the search program TurboSequest. All matching spectra were verified by manual interpretation.
Protease Inhibitors from Phytophthora

Amino acid signal peptide with a significant mean E value of 0.88 and hidden Markov model score of 0.97. Similarity searches of the predicted protein against the nonredundant database of GenBank™ using the BLASTP program (33) revealed two domains similar to InterPro domain IPR002350 for Kazal inhibitors (Fig. 1A). The predicted active site P1, which is central to the specificity of Kazal inhibitors (51, 52), was variable with 10 different amino acids represented (Ala, Asp, Glu, His, Lys, Met, Asn, Arg, Ser, and Thr). Remarkably, half (28 of 56) the P1 residues, including those of EPI1, were aspartate (Asp), an uncommon P1 amino acid in other natural Kazal inhibitors. These results suggest that genes encoding proteins with Kazal domains are diverse and ubiquitous in plant pathogenic oomycetes.

EPI1 Inhibits the Serine Protease Subtilisin A—To determine whether EPI1 functions as a serine protease inhibitor as predicted by bioinformatic analyses, we expressed in E. coli and affinity-purified rEPI1 as a fusion protein with the FLAG epitope tag at the N terminus. Silver staining of the purified rEPI1 fraction after SDS-PAGE revealed a single band indicating high purity. Chymotrypsin, trypsin, and subtilisin A, representing three major classes of serine proteases, were selected for inhibition assays with the purified rEPI1. Protease activity was measured with or without EPI1. In repeated assays, rEPI1 was found to inhibit about 90% of the measured activity of subtilisin A but did not cause apparent inhibition of the other two proteases (Fig. 2A). Time courses of chromogenic substrate hydrolysis by subtilisin A in the presence of increasing amounts of rEPI1 were performed and indicated that rEPI1 inhibition followed a typical dose-response pattern (Fig. 2B). The inhibitory constant (Ki) for subtilisin A inhibition by rEPI1 was determined at 2.77 ± 1.07 nM. These results suggest that epi1 encodes a functional protease inhibitor that specifically targets the subtilisin class of serine proteases.

EPI1 Inhibits BTH-induced Apoplastic Proteases from Tomato—In tomato, some members of the subtilisin-like family P69, namely P69B and P69C, are known to be induced by pathogens and stress treatments and are classified as PR proteins (PR-7 class) (20–22). To test whether rEPI1 inhibits PR-like proteases in tomato, the salicylic acid analog BTH was applied to tomato plants to induce defense-related proteases. In-gel protease assays of tomato leaf intercellular fluid from both H2O-treated and BTH-treated plants revealed that, as expected, BTH induced the production of abundant extracellular proteases in tomato that migrated as two separate but close bands (Fig. 3A). Inhibition assays revealed that rEPI1 dramatically inhibited these BTH-induced proteases as well as partially inhibited these BTH-induced proteases as well as partially inhibited a constitutive protease. The total endoprotease activity was observed and corresponded to 28 and 27% of total activity in control and BTH-treated tomato, respectively (Fig. 1B).

EPI1 Interacts with Pathogenesis-related Subtilases of the Tomato P69 Subfamily—To identify the plant proteases targeted by rEPI1, coimmunoprecipitation was performed on tomato intercellular fluid incubated with rEPI1 using FLAG antibody covalently linked agarose beads. In addition to rEPI1, two proteins were pulled down with the FLAG antibody only in the presence of rEPI1 (Fig. 5A). These two proteins exhibited a...
similar molecular mass of ∼70 kDa (Fig. 5A) and were more abundant in BTH-induced intercellular fluid (Fig. 5B). These results prompted us to test whether these proteins could be tomato P69 subtilisin-like proteases. Western blot analyses with antiserum raised against a peptide specific to P69 subtilisin-like proteases strongly interacted with both bands, suggesting that rEPI1 interacts with P69 subtilases of tomato (Fig. 5B). To confirm the results obtained with the Western blot and further identify which P69 isoforms are the main targets of rEPI1, the two closely migrated protein bands (Fig. 5) were cored from the
The 21 peptides sequenced and perfectly matched the subtilisin-like protease P69B (GenBankTM accession number T07184 or CAA76725) (Fig. 6). Of these 21 peptides, 13 peptides were specific to P69B and did not match any of the other five known P69 isoforms. At this stage it cannot be ruled out that two closely migrated protein bands contain other isoforms, but the results from the tandem mass spectrometry clearly showed that P69B is the main target of rEPI1.

The epil and P69B Gene Are Concurrently Expressed during Infection of Tomato by P. infestans—Expression pattern of both epil1 and P69 genes during infection of tomato by P. infestans was studied by Northern blot and RT-PCR analyses. The epil1 gene displayed the highest mRNA levels 3 days post-inoculation and was moderately up-regulated (approximately 2× based on PhosphorImager quantification) compared with in vitro grown mycelium and relative to the constitutive actA gene (Fig. 7A). Semi-quantitative RT-PCR analyses confirmed these results (Fig. 7B). The expression of P69 protease genes was induced after inoculation with P. infestans and attained the highest level 2 and 3 days after inoculation (Fig. 7A). The expression of P69B gene is the only gene that is up-regulated during colonization of tomato by P. infestans. Total RNA from a time course similar to the one described in A was used in RT-PCR amplifications as described in the text. Amplification of P. infestans elongation factor 2a (Pief2a) and tomato elongation factor 2 (Toef2a) were used as controls to determine the relative expression of epil1 and P69 genes, respectively. C, Western blot analyses of tomato P69 subtilases during colonization by P. infestans. The time course is as described for A. Equal volumes of intercellular fluids were obtained from infected tomato leaves, subjected to SDS-PAGE, and immunoblotted with P69 antisera (α-P69).

Discussion

Plant pathogens manipulate biochemical and physiological processes in their host plants through a diverse array of virulence or avirulence molecules, known as effectors. In susceptible plants, biotrophic plant pathogens produce effectors that promote infection by suppressing defense responses. Here, we describe EP11, a two-domain extracellular protease inhibitor from P. infestans that inhibits apoplastic subtilases of tomato, namely the PR proteins P69. Based on its biological activity and expression pattern, EP11 may function as a disease effector molecule and may play an important role in P. infestans colonization of host apoplast.

Suppression of host defenses is thought to play a critical role in plant-microbe interactions, especially those involving biotrophic pathogens that require live plant cells to establish a successful infection (8, 53). Nonetheless, only a few pathogen molecules that suppress host defenses have been identified. Examples include tomatinase, a saponin-detoxifying enzyme from the fungal pathogen Septoria lycopersici that was recently shown to indirectly suppress host defense responses through its degradation products (9). P. sojae secretes glucanase inhibitor proteins that inhibit a soybean endo-β-1,3-glucanase and are thought to function as counterdefensive molecules that inhibit the degradation of β-1,3/1,6-glucans in the pathogen cell wall and/or the release of defense-eliciting oligosaccharides by host endo-β-1,3 glucanas (54). P. infestans and other Phytophthora species produce water-soluble glucans that suppress induction of host defense responses (10–12). Here, we describe a novel class of pathogen suppressors of plant defense response,
named extracellular protease inhibitors that directly interact with and inhibit host proteases. This interaction could form another type of defense-counterdefense mechanism between plants and microbial pathogens.

We scanned GenBank™ and several other sequence data bases for the occurrence of Kazal-like domains. The examined data sets included the full genome sequence of several plant pathogenic bacteria and fungi. A 235-amino acid protein from tomato/potato, P. soli, P. sojae soybean, and P. halstedii (Table 1). Interestingly, oomycete Kazal motif genes are often expressed during host colonization. Five of the identified sequences were from cdnas obtained from infected plant tissue corresponding to diverse oomycete pathosystems: P. infestans tomato/potato, P. soli, P. sojae soybean, and P. halstedii sunflower. Taken together, the common occurrence of Kazal motifs in several plant pathogenic oomycetes, their in planta expression, and the functional analyses of EPI suggest that inhibition of host proteases could be a conserved virulence strategy among oomycete pathogens. It remains unclear whether other plant pathogenic microbes have evolved inhibitors to counteract plant proteases. If so these inhibitors apparently belong to structural classes other than the Kazal inhibitor domain.

Several plant proteases have been linked to plant defense responses. In tomato, P69 and Rcr3 are two extracellular proteases that have been implicated in the defense response (20, 21, 23). The precise mode of action of these proteases remains unclear. They could degrade secreted proteins from the pathogen, thereby directly contributing to defense. Alternatively, plant proteases could contribute to defense signaling by processing endogenous or pathogen proteins to generate bioactive peptides. Future experiments will focus on determining whether EPI contributes to virulence by protecting other secreted proteins of P. infestans from proteolytic degradation in the host apoplast or by perturbing defense signaling in host plants.

P1 is the primary specificity-determining residue of Kazal inhibitors (51, 52). Remarkably, half (28 of 56) the predicted P1 residues of oomycete Kazal-like inhibitors, including two-thirds (14 of 21) of the P. infestans inhibitor domains, are aspartate. This is an uncommon P1 amino acid in natural Kazal inhibitors of animals and apicomplexans. This striking feature is remarkable in light of a recent finding that two oat proteases with caspase activity and specificity are subtilisin-like serine proteases that have been implicated in the defense response (20, 21, 23). The precise mode of action of these proteases remains unclear. They could degrade secreted proteins from the pathogen, thereby directly contributing to defense. Alternatively, plant proteases could contribute to defense signaling by processing endogenous or pathogen proteins to generate bioactive peptides. Future experiments will focus on determining whether EPI contributes to virulence by protecting other secreted proteins of P. infestans from proteolytic degradation in the host apoplast or by perturbing defense signaling in host plants.

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