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Research paper

Characterization of a novel Kazal-type serine proteinase inhibitor of *Arabidopsis thaliana*

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ABSTRACT

Many different types of serine proteinase inhibitors have been involved in several kinds of plant physiological processes, including defense mechanisms against phytopathogens. Kazal-type serine proteinase inhibitors, which are included in the serine proteinase inhibitor family, are present in several organisms. These proteins play a regulatory role in processes that involve serine proteinases like trypsin, chymotrypsin, thrombin, elastase and/or subtilisin. In the present work, we characterized two putative Kazal-type serine proteinase inhibitors from *Arabidopsis thaliana*, which have a single putative Kazal-type domain. The expression of these inhibitors is transiently induced in response to leaf infection by *Botrytis cinerea*, suggesting that they play some role in defense against pathogens. We also evaluated the inhibitory specificity of one of the Kazal-type serine proteinase inhibitors, which resulted to be induced during the local response to *B. cinerea* infection. The recombinant Kazal-type serine proteinase inhibitor displayed high specificity for elastase and subtilisin, but low specificity for trypsin, suggesting differences in its selectivity. In addition, this inhibitor exhibited a strong antifungal activity inhibiting the germination rate of *B. cinerea* conidia *in vitro*. Due to the important role of proteinase inhibitors in plant protection against pathogens and pests, the information about Kazal-type proteinase inhibitors described in the present work could contribute to improving current methods for plant protection against pathogens.

1. Introduction

Proteinase inhibitors are widely distributed from bacteria to humans. In plants, serine proteinase inhibitors have been described in many species [1] and their physiological role includes the regulation of endogenous proteinases in seed dormancy, mobilization of storage proteins [2] and protection against proteolytic enzymes from bacteria, fungi, parasites and insects [3,4]. The best-known serine proteinase inhibitors are those belonging to the Kazal, Kunitz, Bowman-Birk, serpin and pacifastin families [5].

Plant defense mechanisms through the action of serine proteinase inhibitors involve the inhibition of serine proteinases produced by pathogens, which leads to a reduction in the availability of amino acids required for pathogen growth and development [3,4]. Several reports have shown that serine proteinase inhibitors are able to inhibit fungal or bacterial proteolytic enzymes as well as serine proteinases of insect guts, suggesting that these inhibitors act on a broad range of plant pathogens [6].

Botrytis cinerea is a necrotrophic fungus that affects a wide range of crops, causing significant economic losses [7]. This fungus is currently placed in the second position of the worldwide ranking of the most important phytopathogenic fungi [8]. In addition, *B. cinerea* is considered as a model organism for the study of

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pathogenicity mechanisms of wide-host-range necrotrophic fungi, due to the easiness to obtain spores in laboratory conditions and the availability of molecular tools that allow its genetic manipulation. Interestingly, *B. cinerea* secretes trypsin- and chymotrypsin-like enzymes, which may be involved in the invasion process [9,10].

Kazal-type serine proteinase inhibitors are proteins that have one or multiple Kazal inhibitory domains. Most Kazal-type domains described so far are characterized by six highly conserved cysteine residues, which are able to form three intra-domain disulfide bonds [11,12]. Kazal-type serine proteinase inhibitors play a regulatory role in processes that involve serine proteinases like trypsin, chymotrypsin, subtilisin, thrombin and/or elastase [12]. The first works on Kazal-type serine proteinase inhibitors were performed in vertebrates, particularly mammals and birds [5,13,14]. Subsequently, these inhibitors were characterized in other organisms such as parasites, insects, leeches, crustaceans and fungi [5,15]. Although Kazal-type serine proteinase inhibitors have also been identified in plants [16,17], little is known about their specific roles in these organisms. Thus, the aim of the present work was to characterize two putative Kazal-type serine proteinase inhibitors from *Arabidopsis thaliana*, which we named *A. thaliana* Kazal-type Proteinase Inhibitor 1 (AtKPI-1) and *A. thaliana* Kazal-type Proteinase Inhibitor 2 (AtKPI-2). To contribute to the understanding of these unknown predicted proteins, we evaluated the spatial expression of both genes in *A. thaliana*, and their expression profile after *B. cinerea* infection. Also, we evaluated the proteinase inhibitory activity of a truncated version of the recombinant AtKPI-1 protein and its anti-fungal effect on the germination of *B. cinerea* conidia *in vitro*.

2. Materials and methods

2.1. Sequence analysis

The *AtKPI-1* (gene ID: AT3G61980) and *AtKPI-2* (gene ID: AT4G01575) genes were identified from http://www.arabidopsis.org. The putative Kazal-type proteins were analyzed using the BLASTP program (http://blast.ncbi.nlm.nih.gov/blast) and MotifScan program (http://hits.isb-sib.ch/cgi-bin/PFSCAN). The signal sequence was predicted by the Signal P 4.1 program (http://www.cbs.dtu.dk/services/SignalP). Transmembrane peptides were predicted by the HMMTOP 2.0 program (http://www.enzim.hu/hmmtop/). Sequenced cDNA was compared using the BLAST databanks (http://www.ncbi.nlm.nih.gov/Blast). Sequence analysis was achieved using the blastn, blastx, and blast two-sequence programs (http://www.ncbi.nlm.nih.gov/Blast). Multiple alignments of gene data matrices and protein sequence similarity were calculated using Clustal W [18] and BioEdit Ver. 7.25 [19].

2.2. Plant material and growth conditions

Arabidopsis thaliana Col-0 seeds were germinated in pots containing a perlite/soil/sand mix (1:1:1) and then stratified at 4 °C for 2 days. Afterwards, pots were moved to a glasshouse (23 °C/16 h photoperiod/photon flux density of 200 μmol m s^{-1}). Eight fully developed leaves from 21-day-old plants were used in all the experiments.

2.3. Botrytis cinerea culture and plant inoculation assays

Conidia of *B. cinerea* strain B05.10 were plated on potato dextrose agar medium supplemented with tomato leaves (40 mg/ml). The plates were kept for 7–10 days in the dark at room temperature for sporulation. For plant inoculation, conidia were harvested with sterile water and Tween-20 (0.02%, v/v). Then, they were filtered and counted with a hemocytometer. A concentration of 5×10^5 conidia was used for inoculation. Conidia were resuspended and incubated as described by Flors et al. [20].

For gene expression analysis in response to *B. cinerea* infection, plants were inoculated with the conidial suspension (5×10^5 conidia/ml) as described previously [21]. The size of the necrotic lesions was measured using the Image-Pro®Plus V 4.1 software (Media Cybernetics LP, Maryland, USA). Plants treated with Potato Dextrose Broth (PDB) plus 10 mM sucrose and 10 mM KH_2PO_4 were used as controls. All determinations were performed in duplicate.

2.4. Tissue collection

For the spatial analysis of gene expression in *Arabidopsis* plants infected with *B. cinerea*, leaves, flowers and mature roots from 6-week-old plants, seeds, seedlings and young roots from seedlings were harvested, frozen in liquid nitrogen and stored at − 80 °C until further use.

For the time course of gene expression during the local response of *Arabidopsis* plants to *B. cinerea* infection, leaf disks were cut from inoculated leaves, as previously [21]. To analyze the systemic effects of *B. cinerea* infection on gene expression, tissue samples were taken from non-inoculated leaves younger than inoculated ones. Leaves from plants inoculated with PDB supplemented with 10 mM sucrose and 10 mM KH_2PO_4 were used as controls. Local and systemic leaves were collected 6, 12, 24, 48 and 72 h after inoculation with *B. cinerea*. Each treatment comprised three replicates and each replicate contained three treated leaves. Collected tissues were frozen in liquid nitrogen and stored at - 80 °C until RNA isolation.

2.5. RNA isolation and Real Time PCR

Total RNA was isolated from frozen *A. thaliana* tissue using Trizol reagent (Invitrogen, Massachusetts, USA), following the manufacturer's instructions. The RNA concentration and RNA integrity were analyzed as previously [22]. cDNA was synthesized using M-MLV reverse transcriptase (Promega, Wisconsin, USA) and oligo dT_{20} (Invitrogen) according to the manufacturer's instructions. This cDNA was used as a template for Real Time PCR (qRT-PCR).

The steady-state mRNA levels were analyzed by qRT-PCR as indicated previously [21]. Primer sequences for all the experiments are listed in Supplementary Table 1. Relative quantification was achieved by the comparative cycle threshold method. The ubiquitin gene (*UBQ10*) was used as endogenous control. Reactions were carried out in an Mx3005P qPCR System using the MxPro qPCR Software 4.0 (Stratagene, California, USA). For comparative purposes, relative gene expression was defined with the value of 1 in control plants.

2.6. Cloning of the Arabidopsis thaliana Kazal-type serine proteinase inhibitor gene

The plasmid RAFL 06-08-B11 containing the full-length AtKPI-1 isoform (AT3g61980) provided by RIKEN BRC [23,24] was used as a template for PCR reactions using the following primers: forward primer: 5′caccctcgaggcggatgatttcccagataaatcc 3′; reverse primer: 5′ ctgcagtcattgtccaggaacagaagcacttcc 3′. From the confirmed sequence, a codon-optimized AtKPI-1 sequence was synthesized by GenScript USA Inc. This sequence encodes a truncated version of AtKPI-1 (from aa_{23} to aa_{96}). This AtKPI-$1_{23\text{-}96}$ fragment was digested with *Eco*R1 and *Pst*1 and ligated into the *Escherichia coli* expression cassette pRSFDuet1 (Novagen, Darmstadt, Germany).

2.7. Expression and purification of the Arabidopsis thaliana Kazal-type serine proteinase inhibitor gene

Recombinant N-terminal His-tagged AtKPI-1_{23-96} (rAtKPI-1T) was expressed in *E. coli* BL21 (DE3) and C43 (DE3) cells. Bacteria were grown in LB medium containing 30 mg/l kanamycin, at 37 °C up to a cell mass of 0.5 (OD = 600). Expression was induced with 0.5 mM IPTG for 18 h at 16 °C and cells were harvested by centrifugation and stored at −20 °C until use.

All purification procedures were carried out as previously [22]. Briefly, soluble non-denaturing rAtKPI-1T was purified using a nitrilotriacetic acid-Ni^{2+} column (Qiagen, California, USA) and eluted stepwise with 10, 20, 40, 60 and 250 mM imidazole in lysis buffer. Recombinant AtKPI-1T was stored at 4 °C.

2.8. SDS-PAGE and western blot analysis

Eluted fractions were incubated at 100 °C for 5 min in loading buffer and separated by 18% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) using a Mini-Protean system III (Bio-Rad, California, USA). Then, proteins stained with Coomassie Brilliant Blue were analyzed by MALDI-TOF-TOF as described previously [22]. For western blot analysis, proteins separated by 18% SDS-PAGE were transferred onto PVDF membranes (GE Healthcare, Buckinghamshire, UK) using an Electro-transfer unit (Bio-Rad). The membranes were incubated with anti-mouse tetra-His as first antibody (1:500, Qiagen), and later with anti-mouse IgG conjugated to alkaline phosphatase (1:5.000; Sigma, Missouri, USA) as secondary antibody. The phosphatase activity was measured by the addition of NBT/BCIP substrate. PageRuler TM Pre-stained Protein Ladder (Fermentas, Massachusetts, USA) was used as molecular marker.

2.9. Proteinase inhibitory activity of rAtKPI-1T

Proteinase-inhibitory activity of rAtKPI-1T was demonstrated by gelatin-containing SDS-PAGE, a caseinolytic radial diffusion assay and digestion assays with specific chromogenic blocked *p*-nitroanilide substrates. For trypsin-inhibitory activity, gels were supplemented with 0.15% (w/v) gelatin, as described previously [25]. Porcine trypsin enzyme (28 nM, Sigma) was incubated with or without 0.3, 0.6 and 0.9 μM of rAtKPI-1T in the activity buffer [25] at 0 °C for 60 min and then subjected to electrophoresis. The gels were pretreated as previously [25], and then stained with Coomassie Brilliant Blue.

For elastase and subtilisin-inhibitory activity assays, the polyacrylamide gel mixture was supplemented with 1% (w/v) non-fat powdered milk and poured into molds. Porcine pancreatic elastase and subtilisin enzymes from *Bacillus licheniformis* (130 nM and 120 nM, respectively, Sigma) were separately incubated with or without 0.15, 0.3 and 0.6 μM of rAtKPI-1T in activity buffer [25] at 0 °C for 60 min and then loaded on each solidified casein-containing gel. The positive control inhibitor rTgPI and the negative control rAtHsp81.2 were used at 15 nM and 40 nM, respectively. The gels were incubated at 37 °C overnight.

The residual hydrolytic activity of the above-mentioned serine proteinases was also measured after incubation of each of them with rAtKPI-1T (13.5 μg/μl) in comparison with rTgPI (7.22 μg/μl) as positive control [25] and rAtHsp81.2 (14.2 μg/μl) [22] as negative control, by chromogenic assays [26]. Briefly, each enzyme was incubated with increasing amounts of each recombinant protein for 1 h at 0 °C followed by 10 min at 37 °C. rAtKPI-1T concentrations ranged between 1.0 and 8.7 nM for trypsin, subtilisin and elastase inhibition assays. After addition of substrate to the reaction mixture, the release of p-nitroaniline from the peptidyl p-nitroanilide substrate was measured at 410 nm in a spectrophotometer. The reaction mixtures were prepared as described by Bruno et al. [2004]: for bacterial subtilisin: 3.7 nM enzyme, 100 mM Tris HCl, 150 mM NaCl, 10 mM $CaCl_2$, Triton X-100 0.005% v/v, 0.25 mM CBZ-Gly-Gly-Leu-pNA, pH 8.0, 10 min, 37 °C; for porcine pancreatic elastase: 3.9 nM enzyme, 50 mM Tris HCl, 0.25 mM Suc-Ala-Ala-Ala-*pNA*, pH 7.4, 10 min, 37 °C; and for porcine pancreatic trypsin: 4.3 nM enzyme, 50 mM Tris-HCl, 100 mM NaCl, 0.25 mM Bz-Pro-PheArg-*pNA*, pH 8.0, 0 °C, 5 min. Molar ratios were calculated as previously described [26]. We recorded residual activity (%) after inhibition of rAtKPI-1T and controls rTgPI and rAtHsp81.2 on each enzyme versus the molar ratio (I/Eo).

To study the specificities, each enzyme was pre-incubated with increasing inhibitor concentrations, times and incubation time, as described above. The reaction was initiated with the specific substrate in each case. The resulting steady-state velocities were measured. The values corresponding to the quotient of the velocities in the presence and in the absence of the inhibitor, respectively, at growing amounts of inhibitor were graphed and Ki values were obtained [27].

Bacterial subtilisin and porcine pancreatic elastase were acquired from Sigma, while porcine pancreatic trypsin was purchased from Life Technologies, New York, USA.

2.10. Conidial germination assay

To determine the inhibitory activity of recombinant proteinases on the germination of *B. cinerea* conidia, 286 nM of rAtKPI-1T and 83 nM of rTgPI were dissolved in 10 mM Tris-HCl and mixed with 20 μl of a conidial suspension (1.8×10^5 conidia/ml in 25 mM potassium phosphate buffer, pH 5 and 2 M glucose). Volume was made up to 75 μl with 25 mM potassium phosphate buffer pH 5. The mixture was incubated at 25 °C in a well of a 96-well plate (Immuno Plate Maxisorp; Nunc, New York, USA). After incubating for 3, 6 and 9 h, germination rates were determined using a binocular microscope (Nikon Diaphot-tmd, Tokyo, Japan). Conidia were considered to be germinated when the length of the germ tube was at least twice the length of the conidia. More than 100 conidia were counted per well, and four replicate wells were analyzed. The experiment was conducted three times. Eluates from non-transformed *E. coli* BL21 (DE3) dissolved in 10 mM Tris-HCl were used as control.

2.11. Statistical analysis

Statistical analysis was carried out with the Prism 5.0 Software (GraphPad, California, USA) using one-way or two-way analysis of variance (ANOVA) and the Bonferroni's multiple comparison Test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Sequence analysis of Kazal-type proteinase inhibitors of Arabidopsis thaliana

Previous comparative genomic analyses have allowed identifying several peptidase inhibitors in plants [16,17]. Those analyses showed two putative intronless genes that encode for putative Kazal-type serine proteinase inhibitors: AT3g61980 and AT4g01575, which we named AtKPI-1 and AtKPI-2, respectively.

AtKPI-1 has a 354-bp ORF encoding for a putative protein of 117 amino acids (AtKPI-1), whereas *AtKPI-2*has a 435-bp ORF encoding for a putative protein of 144 amino acids (AtKPI-2). The predicted amino acid sequences revealed 47% identity between AtKPI-1 and AtKPI-2 (Fig. 1A). Using the Signal P 4.1 program, we also identified

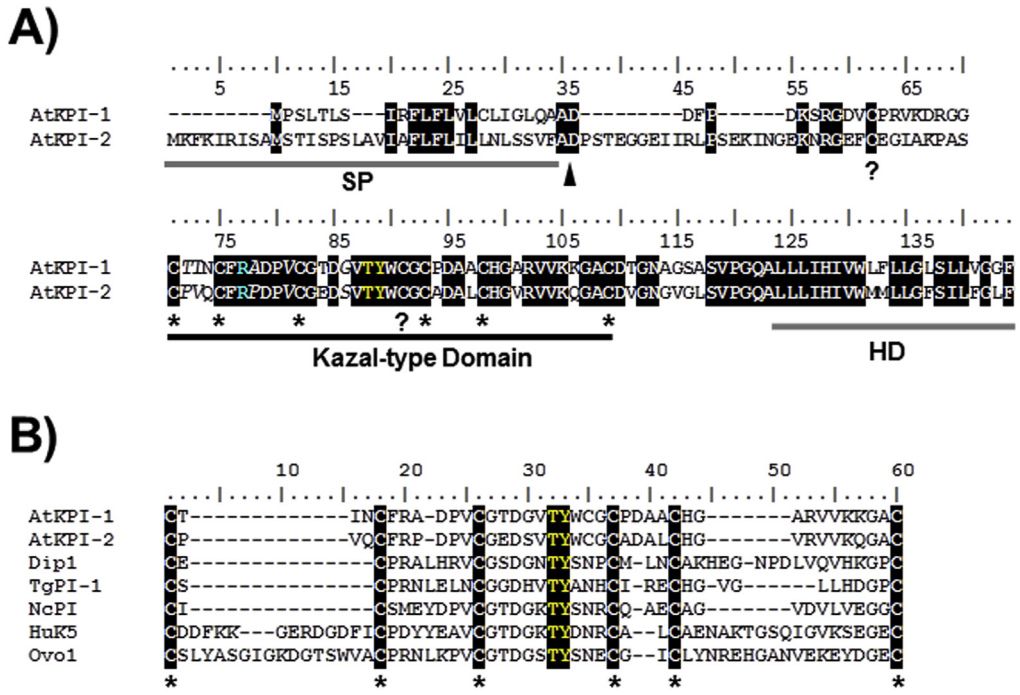

Fig. 1. (A) Amino acid sequence alignment of putative Kazal-type proteinase inhibitors found in *Arabidopsis thaliana*. AtKPI-1 and AtKPI-2 have a single putative Kazal-type domain (black underline), a signal sequence at the N-terminus (SP) and a hydrophobic domain at the C-terminus (HD) (gray underline). The putative Kazal-type domains were identified showing the presence of six highly conserved cysteine residues (*). The other two cysteines present in both plant sequences are indicated with a question mark (?). The arginine (blue) which composes a putative P1 site of the reactive loop and other aligned residues that may be involved in the specificity of these inhibitors (italics) are also indicated. (B) Multiple sequence alignment of the Kazal domain from *A. thaliana* (AtKPI-1 and AtKPI-2), *Homo sapiens* (HuK5), *Gallus gallus* (Ovo1), *Dipetalogaster maximus* (Dip1), *Neospora caninum* (NcPI) and *Toxoplasma gondii* (TgPI-1). The conserved threonine-tyrosine residues between cysteine 3 and 4 (yellow) are indicated.

that both proteins contain a single putative signal sequence at the N-terminal end, suggesting that these proteins would be directed to the apoplast. Finally, using the HMMTOP 2.0 program, we identified a potential transmembrane domain/hydrophobic region involving the last 22 amino acids at the C-terminal end of both proteins (Fig. 1A). As previously described by Santamaria et al. [17], both putative proteins have a single Kazal-type domain (Fig. 1A and B) also detected by the MotifScan program. In addition, the analysis of the multiple sequence alignment showed that *A. thaliana* putative Kazal-type serine domains aligned with other well-known Kazal-type domains (Fig. 1B) and that six cysteine residues and the threonine-tyrosine residues are highly conserved among different species (Figs. 1B and S1). It can also be observed that other two cysteine residues are conserved in both *A. thaliana* putative Kazal-type serine sequences: one of them is located into the inter-putative cysteine 3 and 4 spacing of the putative Kazal-type domain (Fig. 1A and B). Based on multiple sequence alignment, we also identified an arginine residue, which could be a putative P1 site of the reactive loop [28], located two residues after the putative second cysteine (Figs. 1A and S1). Moreover, the arginine residue placed in the second position after the putative second cysteine residue is highly conserved among putative Kazal-type domains of other plant species (Fig. S1).

3.2. Expression pattern of the AtKPI-1 and AtKPI-2 genes in different tissues of A. thaliana

A qRT-PCR was performed to analyze the expression of *AtKPI-1* and *AtKPI-2* in different tissues. Basal levels of the transcripts for both Kazal-type genes were measured in young and mature roots, leaves, flowers, seeds and seedlings (Fig. 2). The expression of *AtKPI-1* and *AtKPI-2* was relativized to the ubiquitin gene (*UBQ10*),

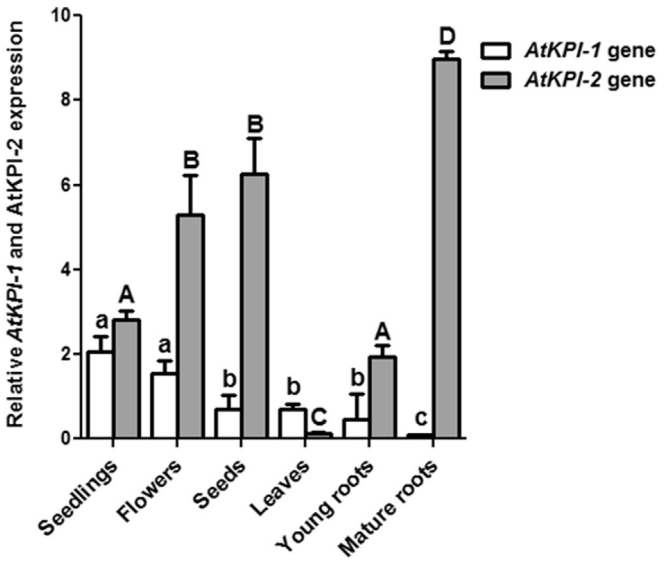

Fig. 2. Basal transcript levels for both Kazal-type genes in different tissues of *Arabidopsis thaliana* plants. qRT-PCR was used to analyze the abundance of *AtKPI-1* and *AtKPI-2* transcripts in *A. thaliana*. Results are expressed as relative to *UBQ10* mRNA in each tissue and are presented as means ± SEM of five replicates. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's multiple comparison Test. Different letters indicate statistically significant differences ($p < 0.01$). Lower case letters correspond to statistical analysis of the *AtKPI-1* gene. Capital letters correspond to statistical analysis of the *AtKPI-2* gene.

which was used as housekeeping transcript. Fig. 2 shows that both genes presented a differential expression pattern among different plant tissues. *AtKPI-1* was mainly expressed in seedlings and

flowers and was undetectable in mature roots (Fig. 2), whereas *AtKPI-2* was strongly expressed in seeds, mature roots and flowers, and was weakly detected in leaves (Fig. 2). Likewise, *AtKPI-2* was more strongly expressed than *AtKPI-1* in flowers, roots and seeds, while both genes were similarly expressed in seedlings. Conversely, *AtKPI-1* expression was 5-fold higher than *AtKPI-2* expression in leaves (Fig. 2).

3.3. Time course of AtKPI-1 and AtKPI-2 gene expression after Botrytis cinerea infection

To elucidate the potential involvement of *AtKPI-1* and *AtKPI-2* in plant defense, we characterized the expression of these genes after the interaction of *A. thaliana* with *B. cinerea*. To this end, leaves of *A. thaliana* plants were inoculated with *B. cinerea* and the transcript levels of both genes were analyzed in local and systemic leaves by qRT-PCR for 72 h (Fig. 3).

Mycelial growth and the development of necrotic tissue at the inoculation site were clearly visible as early as 24 h after inoculation (Fig. S2A). In addition, the expression of the *PDF1.2* gene was evaluated as a marker of Jasmonic acid/Ethylene-dependent plant response. This marker gene was significantly induced upon *B. cinerea* infection (Fig. S2B). At local level, *AtKPI-1* mRNA levels amounts were significantly increased 24 h after *B. cinerea* inoculation, while *AtKPI-2* mRNA levels showed no significant variation (Fig. 3A). On the other hand, *AtKPI-1* mRNA levels of systemic leaves from inoculated plants were similar to those detected in uninfected plants, whereas the expression levels of *AtKPI-2* mRNA levels were significantly increased at 24 h (Fig. 3B).

3.4. Recombinant expression and purification of AtKPI-1

To know the biochemical properties of *A. thaliana* Kazal-type inhibitors, we planned their expression as recombinant proteins in bacteria. Different approaches, such as the use of different *E. coli* strains and temperatures for bacterial growth and induction of recombinant protein expression, codon optimization and elimination of the C-terminal hydrophobic region were developed. We determined the appropriate host for an efficient expression of recombinant proteins by testing its expression in *E. coli* BL21 (DE3) and C43 (DE3) strains. The latter is commonly used to overcome the toxicity associated with overexpression of toxic proteins [29]. Since hydrophobic regions could affect the expression of recombinant proteins and interfere with the purification protocol, the signal peptide and the C-terminal hydrophobic region of the full-length AtKPI-1 and AtKPI-2 were eliminated and truncated versions of these proteins were generated. However, successful recombinant protein expression was achieved only for AtKPI-1 (Fig. 4A, B). After addition of IPTG, no expression of the recombinant protein was detected in the C43 (DE3) strain (Fig. 4A), while a protein with apparent molecular mass of 12 kDa was clearly detected in BL21 (DE3) (Fig. 4B).

The presence of the recombinant AtKPI-1T$_{23\text{-}96}$ (rAtKPI-1T) eluted from the affinity chromatography column at different imidazole concentrations was confirmed by SDS–PAGE under reducing conditions and western blot analysis (Fig. 4C, D). A major protein band of approximately 12 kDa was eluted with 250 mM imidazole (Fig. 4C). The presence of rAtKPI-1T was confirmed by western blot using the anti-His antibody (Fig. 4D). Other bands reacted with the anti-His antibody, and all these bands were excised from the gel and analyzed by MALDI-TOF/TOF. This analysis revealed that four peptides (70% sequence coverage) from these bands matched with the AtKPI-1T sequence, indicating that the bands with molecular weights higher than ~12-kDa corresponded to oligomeric forms of the recombinant protein (Fig. S3). In addition, total protein extracts from leaves, flowers and seedlings were

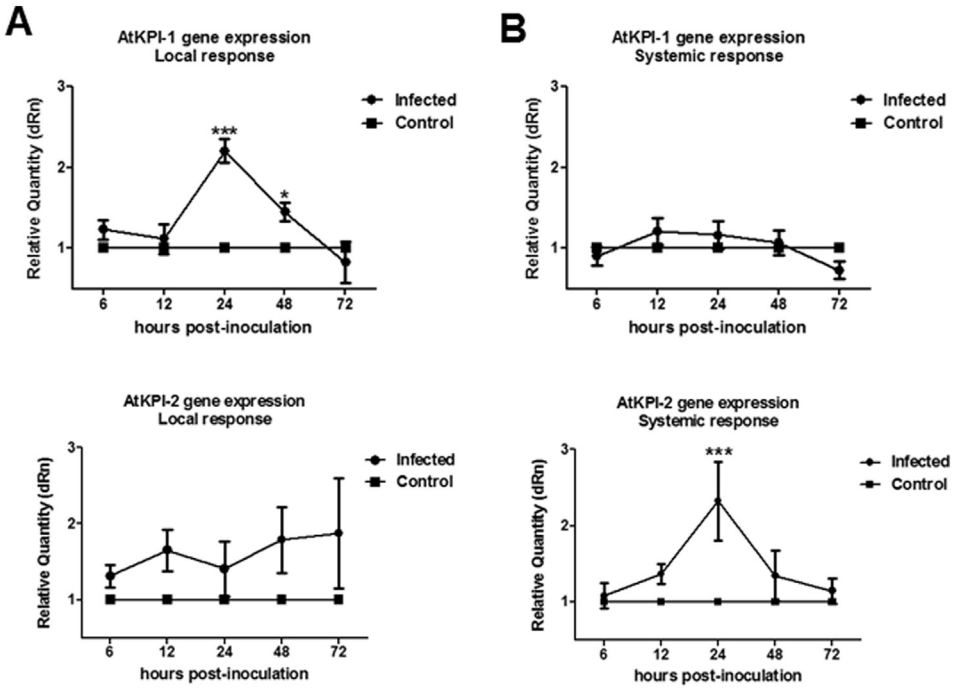

Fig. 3. Time-course analysis of the *AtKPI-1* and *AtKPI-2* genes in leaves of *Arabidopsis thaliana* plants infected with *Botrytis cinerea* at local (A) and systemic (B) level. RT-PCR analysis with equal cDNAs was performed to detect *AtKPI-1* and *AtKPI-2* as described in Material and Methods. Tissues obtained from mock-inoculated leaves were used as controls. For each time after treatment (6, 12, 24, 48, 72 h), relative levels of expression for both genes are shown. Results are expressed as relative to *UBQ10* and normalized respect to mock-inoculated plants (control), which were assigned to 1. Results are means of three replicates ± SEM and are representative of three experiments. Statistical analysis was performed by one-way ANOVA using the Bonferroni's Multiple Comparison Test. ***$p < 0.001$.

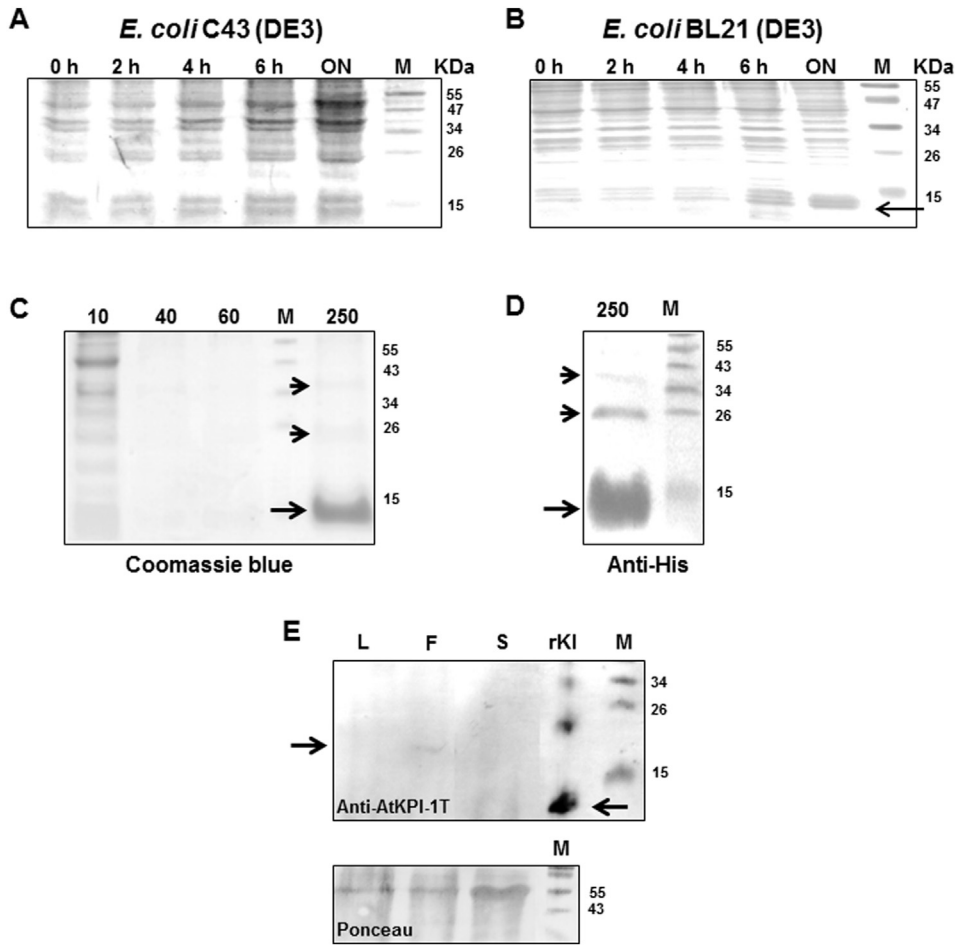

Fig. 4. Expression and purification of the truncated version of the recombinant AtKPI-1 (rAtKPI-1T). (A) Expression analysis of rAtKPI-1T in BL21 (DE3). (B) Expression analysis of rAtKPI-1T in C43 (DE3). Total soluble proteins extracted from bacteria before induction (0 h) and after 2, 4, 6 h and overnight (ON) induction with 0.5 mM IPTG were electrophoresed on SDS–PAGE gels and stained by Coomassie Brilliant Blue. The arrow indicates the rAtKPI-1T protein. (C) Purification of the recombinant protein was checked in a Coomassie blue-stained polyacrylamide gel. rAtKPI-1T was eluted using a Ni^{+} affinity chromatography column. Imidazole concentrations (mM) used for elution are indicated above the respective lane. (D) Western blot analysis of the fraction eluted with 250 mM imidazole using anti-His_6 antibody. Arrows indicate the bands analyzed by MALDI-TOF/TOF. E) Expression of AtKPI-1 in flowers, leaves and seedlings of *A. thaliana*. Reduced samples containing 75 μg of total protein from leaf (L), flower (F) and seedling (S) extracts were separated by 18% SDS-PAGE and then transferred onto a PVDF membrane. The membrane was incubated with mouse anti-AtKPI-1T polyclonal antibodies (1:500), followed by anti-mouse IgG phosphatase alkaline-conjugate secondary antibodies (1:5000). The bands were detected as described in Material and Methods. rKI: rAtKPI-1T protein. M: pre-stained molecular weight protein marker (Fermentas). The membrane was stained by ponceau staining as control of the protein loading.

used for expression analyses. Western blot analysis revealed a protein band that was immune-detected with mouse polyclonal anti-rATKPI-1T antibodies in extracts from flowers as well as with rAtKPI-1T (Fig. 4D).

3.5. Inhibitory activity of rAtKPI-1T

We next investigated whether AtKPI-1, by using rAtKPI-1T, has functions similar to those of other Kazal-type serine proteinase inhibitors and exhibits inhibitory activities against serine proteinases. Proteinase inhibitory activity of rAtKPI-1T was analyzed by radial caseinolytic assay and gelatin-containing SDS-PAGE (Fig. 5). Porcine elastase and bacterial subtilisin generated a dark circle against a white background, indicating proteinase activity (Fig. 5A and B). After incubation in the presence of rAtKPI-1T, the caseinolytic activity was totally inhibited (Fig. 5A, B and D). On the other hand, the proteinase activity of porcine trypsin was detected by a clear band against a dark blue background (Fig. 5C). After incubation with rAtKPI-1T, the gelatinolytic activity was partially eliminated (Fig. 5C and D). A recombinant Kazal-type serine proteinase inhibitor of *Toxoplasma gondii* (rTgPI) was also incubated with the serine proteinases and used as a positive control [25]. The incubation with rTgPI totally abolished the proteinase activity in all cases (Fig. 5). The recombinant 90-kDa heat shock protein of *A. thaliana* (rAtHsp81.2) [22], purified with the same procedure, was incubated with the proteinases and used as a negative control. No inhibition was observed in any case by using rAtHsp81.2 (Fig. 5). To determine the specificity of rAtKPI-1T on the different proteinases, Ki values were calculated from kinetics graphics as vi/vo vs inhibitor concentration by using a more sensitive digestion assay with specific chromogenic substrates. The estimated Ki values were 3.3, 5.2 and 6.8 for elastase, subtilisin and trypsin, respectively (Table 1). In addition, we compared the residual activities of each enzyme after incubation with the inhibitor rAtKPI-1T (%) vs the molar ratio I/Eo (Fig. 6). rTgPI and rAtHsp81.2 were used as positive and negative controls, respectively. While increasing the molar ratio between rAtKPI-1T and each enzyme, the inhibitory activity of rAtKPI-1T reached a maximum towards each proteinase, up to 70, 40, and 20% for elastase, subtilisin, and trypsin, respectively (Fig. 6). rAtKPI-1T inhibited elastase, subtilisin and trypsin in a much lower degree than the control rTgPI. In this sense, rTgPI showed total inhibition at molar ratios = 1 and 2 for elastase and trypsin, as well

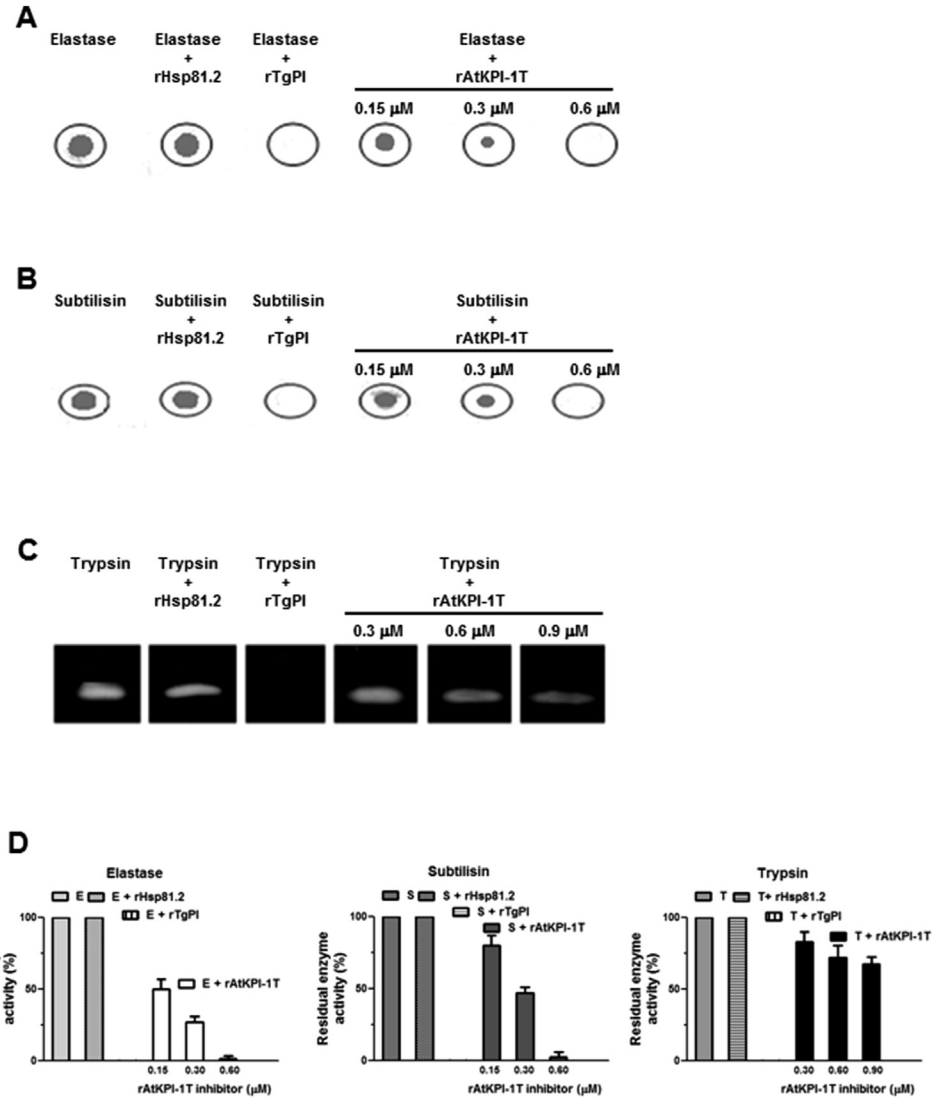

Fig. 5. Analysis of the inhibitory effect of rAtKPI-1T on elastase, subtilisin and trypsin by protein digestion assays. (A) Elastase-inhibitory activity of rAtKPI-1T was analyzed by radial caseinolytic assay. All molds were loaded with 130 nM of elastase. (B) Subtilisin-inhibitory activity of rAtKPI-1T was analyzed by radial caseinolytic assay. All molds were loaded with 120 nM of subtilisin. Caseinolytic activity of elastase and subtilisin was visualized by direct observation of the substrate hydrolysis. (C) Trypsin-inhibitory activity of rAtKPI-1T was analyzed by gelatin substrate-SDS-PAGE. All lanes contained 28 nM of trypsin. rTgPI and rAtHsp81.2 were used as positive and negative controls, respectively. Equal volumes of rTgPI (15 nM), rAtHsp81.2 (42 nM) or different concentrations of rAtKPI-1T were used. After incubation, gels were stained with Coomassie Brilliant Blue. (D) Densitometry of all the radial spots of elastase and subtilisin and gel electrophoresed bands of trypsin was performed by quantitation of intensity with a FujiLAS1000 densitometer equipped with IMAGE GAUGE 3.122 software (FujiFilm, Tokyo, Japan).

Table 1
Specificity of the Kazal-type inhibitor of *Arabidopsis thaliana* (rAtKPI-1T) on serine proteases.

Enzyme	Concentration (nM)	Species	Ki (nM)
Trypsin	4.3	Porcine	6.8
Subtilisin	3.7	Bacterial	5.2
Elastase	3.9	Porcine	3.2

as almost complete inhibition of subtilisin in the range tested. As expected, rAtHsp81.2 did not show specificity for these enzymes. Thus, the absence of inhibition with the recombinant protein used as a control for the purification procedure confirmed a genuine inhibition effect.

3.6. *In vitro* inhibition of *B. cinerea* conidia germination by rAtKPI-1T and rTgPI

To investigate whether rAtKPI-1T and rTgPI are able to exert antifungal effects, we analyzed the germination of *B. cinerea* conidia in culture medium supplemented with recombinant proteinase inhibitors (Fig. 7). After incubation for 6 and 9 h, the germination rates of *B. cinerea* conidia in the medium containing recombinant inhibitor proteins were significantly lower than those in medium without proteinase inhibitors. An inhibition higher than 90% was evident 9 h post-incubation for conidia incubated with rAtKPI-1T or rTgPI (Fig. 7A). Moreover, microscopic analysis showed that germ-tube growth was clearly inhibited by rAtKPI-1T and rTgPI, as compared to control, which showed normal germ-tube growth (Fig. 7B).

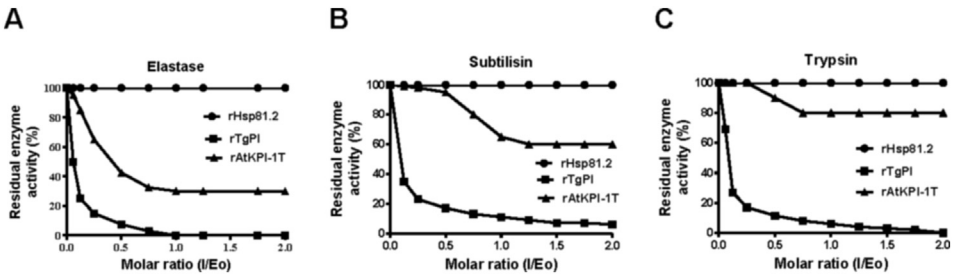

Fig. 6. Inhibitory effect of rAtKPI-1T on elastase, subtilisin and trypsin. A) porcine pancreatic elastase, B) subtilisin from *Bacillus licheniformis*, and C) porcine pancreatic trypsin were used as serine proteinases. rTgPI and rAtHsp81.2 obtained by the same purification method were used as controls. rAtKPI-1T, rTgPI and rAtHsp81.2 from 1.0 to 8.7 nM were confronted with each enzyme. Results are expressed as the percentage of residual activity of each enzyme versus the molar ratio between the concentrations of the recombinant inhibitor used and the concentration of the enzyme in the absence of inhibitor (I/Eo).

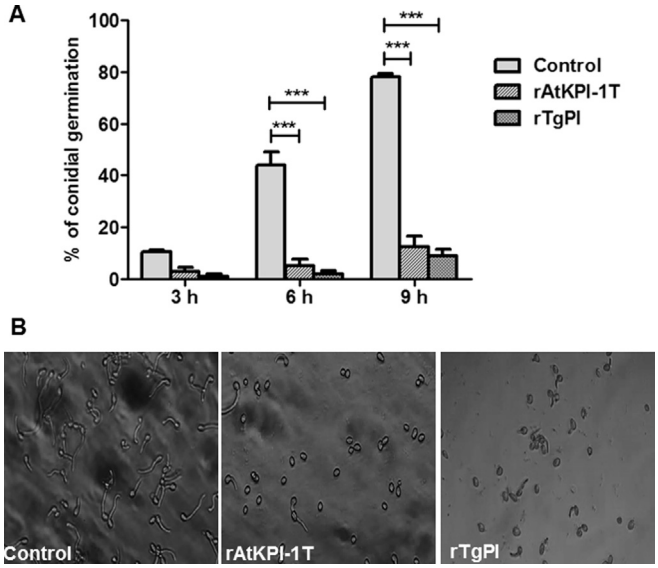

Fig. 7. Germination of *Botrytis cinerea* in medium supplemented with recombinant proteinase inhibitors. (A) Inhibition of conidial germination. Results are means of four replicates ± SEM and are representative of three experiments. Statistical analysis was performed by one-way ANOVA using the Bonferroni's Multiple Comparison Test. ***$p < 0.001$. (B) Germ-tube growth of *B. cinerea* conidia after incubation for 9 h *in vitro*. Control: addition of an eluate from *E. coli* BL21 (DE3); rAtKPI-1T: addition of 286 nM of rAtKPI-1T; rTgPI: addition of 83 nM of rTgPI.

4. Discussion

Proteinase inhibitors are grouped in different family proteins with different biological roles such as cell homeostasis and defense, among others [11,12,30]. Besides, overexpression of serine proteinase inhibitors in plant cells improves the expression of heterologous proteins in plants [31]. In the present study, we characterized two putative genes that encode for two Kazal-type serine proteinase inhibitors in *A. thaliana* [16,17]. Neither of these genes have introns, similarly to other serine proteinase inhibitor genes of plants [32–34]. It has been suggested that the absence of introns in some genes is evolutionarily conserved because it provides a selective advantage, since no splicing is required and the mature RNA is quickly accumulated in the cytoplasm and translated to protein [34]. This could be an advantage for serine proteinase inhibitors, which are quickly required in defense mechanisms against certain pathogens.

According to the inter-cysteine spacing, Kazal-type domains can be classified into classical and non-classical Kazal domains [35]. Classical Kazal domains have a general amino acid sequence of $CX_{6-9}CX_7CX_6YX_3CX_{2-3}CX_{17}C$. Based on this nomenclature and taking into account the analysis of the multiple sequence alignment, the two plant proteins are similar to the Kazal-type serine proteinase inhibitors from other organisms and possess a single putative Kazal-type domain ($CX_3CX_3CX_6YX_3CX_4CX_{10}C$), which would belong to the family of non-classical Kazal domains. At the same time, Kazal-type inhibitors can be also denoted as typical or atypical Kazal domains. Typical Kazal domains contain six cysteine residues forming three disulfide bonds [5,36], while atypical Kazal domains lack the third and sixth cysteines and have two disulfide bonds [14,36,37]. However, a new sub-family of Kazal-type inhibitors, which belong to the non-classical Kazal and contain four disulfide bonds [38,39], three typical bonds of Kazal-type inhibitors plus an additional disulfide bond [39], has been characterized. AtKPI-1 and AtKPI-2 contain six highly conserved cysteines; however, as it has also been noted for these new non-classical Kazal inhibitors, both plant sequences show the presence of other two cysteines. One of them is located eight residues upstream of the putative cysteine 1, while the other cysteine is part of the inter-putative cysteine 3 and 4 spacing. According to the alignment analysis, it is noteworthy that this region between the putative cysteines 3 and 4 contains a highly conserved inter-cysteine space (10 residues) among Kazal-type domains also present in AtKPI-1 and AtKPI-2. In both plant sequences, this region retains the aspartic acid and threonine-tyrosine but not asparagine/serine, which are highly conserved in other Kazal-type domains [40,41]. The presence of these two cysteines in AtKPI-1 and AtKPI-2 may involve other possibilities of forming disulfide bridges and therefore of inducing structural changes. In this case, they should be considered as atypical Kazal–type domains. Further studies should be done to shed light on the exact structure and disulfide bridge present in these two proteins.

The main role of Kazal-type and other serine proteinase inhibitors is to limit and control the spread of serine proteinase activity [42]. The large abundance of different types of serine proteinase inhibitors described in plants suggests that they are involved in many kinds of biological processes [43]. In our study, qRT-PCR analysis showed that AtKPI-1 and AtKPI-2 expression is spatially regulated. The *AtKPI-1* gene was mainly expressed in seedlings and flowers, where also AtKPI-1 protein was detected by western blot. The *AtKPI-2* gene was strongly expressed in seeds, mature roots and flowers. The analysis comparing the expression of both genes showed that *AtKPI-2* transcript levels were higher than those of *AtKPI-1* in most of the tissues analyzed, except in leaves, where *AtKPI-1* was mainly expressed. Plants constitutively express serine proteinase inhibitors in aerial tissues (leaves and flowers) and storage organs (seeds and tubers) as a mechanism to control endogenous proteinase activity during seed dormancy and germination, which suggests that they may act as regulators of nutrient

mobilization and storage of proteins [44]. Future studies are in progress to establish the role of these Kazal-type inhibitor proteins in plant physiology and/or development.

Several reports have suggested that serine proteinase inhibitors can inhibit exogenous serine proteinases as a defense mechanism against insects, fungi, bacteria and mechanical wounding [43,45–47]. In particular, some phytopathogenic fungi secrete extracellular serine proteinases that play an important role in the infection process [48]. In response to these fungal proteinases, plants synthesize proteinase inhibitors that can abolish the enzyme activity [49]. The expression of several serine proteinase inhibitors is induced after inoculation with fungi like *B. cinerea*, and in some cases, these inhibitors have anti-fungi activity [47,49–51]. In the present work, AtKPI-1 expression was moderately increased in infected leaves 24 h post-inoculation, and then decreased to levels similar to those observed in non-inoculated leaves. During the systemic response of non-inoculated leaves of pathogen-infected plants, the expression levels of AtKPI-2 were significantly increased 24 h post-inoculation, as compared to similar leaves from non-inoculated plants. Huang et al. [49] tested the antimicrobial activity of the Kunitz inhibitor of *Nicotiana tabacum* (NtKTI1) against various fungi and bacteria. These authors found that NtKTI1 inhibits mycelial growth of several fungi, and detected the most prominent antifungal activity 24 h post-inoculation. The induction of AtKPI-1 in response to *B. cinerea* infection suggests that this inhibitor could be part of an early local response of *A. thaliana* probably directed to the inhibition of extracellular proteinases produced by this phytopathogen. However, the possibility that AtKPI-1 also modulates the activity of endogenous proteinases that promote disease-associated cell death cannot be ruled out. Conversely, AtKPI-2 is induced systemically, probably as a general response of the plant to maintain cell homeostasis by controlling the endogenous proteinase activity and/or blocking pathogen spread. However, further studies would be necessary to elucidate the role of these putative Kazal-type inhibitor proteins in pathogen defense.

B. cinerea secretes trypsin and chymotrypsin-like proteinases that have been proposed to be involved in the degradation of host cell walls [9,52]. In addition, genomic analysis of *B. cinerea* has allowed the identification of two genes that encode secreted subtilisin proteinases [10]. In this context, we evaluated the inhibitory specificity of AtKPI-1, which showed to be induced during local responses to *B. cinerea* infection. For this purpose, we expressed a truncated version of AtKPI-1 (rAtKPI-1T) in *E. coli* BL21 (DE3). Interestingly, rAtKPI-1T showed high specificity for elastase and subtilisin, but low specificity for trypsin. In agreement with the Ki values obtained, while increasing the molar ratio between rAtKPI-1T and each enzyme, the inhibitory activity of rAtKPI-1T reached a maximum towards each proteinase, up to 70, 40, and 20% for elastase, subtilisin, and trypsin, respectively, signing it as a reversible tight binding inhibitor [27,53]. On the other hand, the inhibitory mechanism of the Kazal-type inhibitor is explained by the 'standard' mechanism, where the inhibitor on the reactive loop binds to the reactive site of the enzyme through a substrate-enzyme-like manner [54]. During this interaction, there is a reversible equilibrium in which the inhibitory complex is able to produce both intact and cut forms of the inhibitor. The latter are the product of the cleavage of the reactive loop by serine proteinases [55]. In this sense, the alignment sequence analysis suggests that the arginine residue could be part of the reactive loop determining the inhibitory specificity. The inhibitory specificity is governed by an amino acid that is called the P1 site of the reactive loop, which resides at the second amino acid downstream of the second conserved cysteine residue. In general, if the P1 amino acid is arginine, the Kazal-type serine proteinase inhibitor strongly inhibits trypsin [5,56]. Several Kazal-type domains with a P1 arginine are able to inhibit not only trypsin but also other serine proteinases like plasmin, thrombin, elastase and subtilisin [56]. Based on that mentioned above, i.e. the presence of two other cysteines, possibly responsible for the formation of disulfide bonds, and the non-classical inter-cysteine spacing in both plant sequences, further experimental evidence about the identity of the P1 residue in the reactive loop would contribute to evaluating the possibility that other residues could influence the degree and specificity of the plant Kazal-type domain inhibition.

Finally, we evaluated the inhibitory activity of rAtKPI-1T on the germination and germ-tube growth of *B. cinerea* conidia. Our results showed that rAtKPI-1T has strong antifungal activity inhibiting conidial germination rate. The specificity profile observed for rAtKPI-1T suggests that potential serine proteinases secreted by *B. cinerea* could be targeted by this inhibitor, leading to the inhibition of conidial germination.

5. Conclusion

AtKPI-1 showed highest expression in seedlings and flowers, while AtKPI-2 showed highest expression in seeds, mature roots and flowers. Both genes are temporally induced during infection with *B. cinerea*. In addition, rAtKPI-1T showed strong inhibitory activity against conidial germination, suggesting that both genes could play some role in plant defense against pathogens. Among the potential targets, rAtKPI-1T showed the strongest inhibition on elastase, followed by subtilisin and trypsin. Further research based on the use of transgenic *Arabidopsis* over-expressing the *AtKPI-1* and/or *AtKPI-2* genes identified and characterized in the present work would contribute to confirming the role of these putative Kazal-type serine proteinase inhibitors in defense responses, and could also contribute to developing novel strategies to improve plant resistance to pathogens.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2016.02.002.

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