PLPKI: A novel serine protease inhibitor as a potential biochemical marker involved in horizontal resistance to *Phytophthora infestans*

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Abstract

Potato leaves infected with *Phytophthora infestans* produced a serine protease inhibitor (PLPKI) with specificity for microbial proteases. Sequencing of the first twenty residues at the NH_2-terminus of the mature PLPKI polypeptide demonstrated that PLPKI is a novel member of the potato protease inhibitor I family. PLPKI inhibited the activity of extracellular proteases produced by two pathogens of potato, *P. infestans* and *Rhizoctonia solani*, but was inactive against proteases secreted into the culture media by the binucleate *Rhizoctonia* N2, a non-pathogenic fungus for potato. Western blot analysis showed a positive correlation between the levels of PLPKI and the degree of horizontal resistance, showing its highest accumulation in clone OKA 5632.11, which has been described as highly resistant. This correlation, together with the ability of PLPKI to completely abolish the secreted serine protease activity of *P. infestans*, suggests that PLPKI may have an active role in protecting potato plants from this pathogenic oomycete and that it could be used as a suitable biochemical marker to help breeders in the selection of cultivars with high degree of horizontal resistance.

Many phytopathogenic microorganisms secrete active extracellular proteases that, along with other enzymes, contribute to their aggressiveness when colonizing plant tissues (Movahedi and Heale 1990, Ball et al. 1991, París and Lamattina 1999). Among them, serine proteases predominate and they can be divided into trypsin-like and subtilisin-like enzymes. In this scenario, plant ability to inactivate proteases derived from phytopathogens may significantly contribute to the plant defence mechanism. Plant protease inhibitors (PIs) are small proteins induced by wounding, insect feeding or microbial attacks that are capable of suppressing enzymatic activity of phytopathogenic microorganisms. They are divided into eighty-five families, based on amino acid sequence homology (according to MEROPS database), or into four classes (serine, cysteine, metallo- and aspartyl) depending on the mechanistic type of proteases which they interact with (Rawlings et al. 2004, 2010). The temporal and spatial patterns of protease inhibitor gene expression, the ability to inhibit pathogen proteases and the enhanced resistance to herbivores of transgenic plants expressing protease inhibitor genes suggest an important role for protease inhibitors in plant defence (Heitz et al. 1999, Hermosa et al. 2006, Turrá et al. 2009, Dunse et al. 2010). However, their clear function in defence responses has been reported only in a limited number of cases.

Much of the work on protease inhibitors from plant origin has been focused on the inhibitors of serine proteases (EC 3.4.21.14). Increases in the levels of protease inhibitor mRNAs, proteins and activities have been demonstrated in several plant–herbivore and plant–pathogen interactions, including enzymes corresponding to the potato inhibitors I and II, Bowman-Birk protease inhibitors and Kunitz trypsin inhibitor families (Geoffroy et al. 1990, Pautot et al. 1991, Rohmeier and Lehle 1993, Ishikawa et al. 1994, Hermosa et al. 2006, Revina et al. 2008, Tamhane et al. 2009, Turrá et al. 2009, Turrá and Lorito 2011).

Potato is a host for many pathogens that affect all organs of the plant and cause reductions in tuber quality and production yield. Among them, the oomycete *P. infestans* cause potato late blight, one of the most detrimental diseases that affect this crop. Therefore, the development of new cultivars with higher degree of resistance to economically important pests and diseases is one of the top priorities for potato breeding programmes worldwide. Traditionally, two forms of genetic resistance to *P. infestans* have been described in potato species: vertical resistance (VR) and horizontal resistance (HR). VR is characterized by interactions between the products of dominant resistance (*R*) genes in the host and corresponding avirulence genes in the pathogen (Umaerus and Umaerus 1994). In contrast, HR is assumed to be based on multiple genes, more durable and, hence, a commercially more attractive form of resistance than vertical resistance (Wastie 1991).

In our laboratory, a proteinase K inhibitor (PLPKI: potato leaf proteinase K inhibitor) that was induced in leaves from a potato cultivar with high degree of horizontal resistance after infection with *P. infestans* (*Pi*), has been purified and characterized. This inhibitor was highly active against proteinase K (EC 3.4.21.14) and less effective on two serine proteases of animal origin, trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1). Interestingly, PLPKI was differentially expressed in two potato cultivars with different degrees of horizontal resistance to *Pi* (Feldman et al. 2000).

The aim of this work was to determine the identity of PLPKI and its relation to other plant serine protease inhibitors and to characterize its activity against extracellular proteolytic activities secreted by two potato pathogens, *Pi* and *R. solani* (*Rs*). Additionally, we wished to establish whether there was a correlation between the degree of HR and the accumulation of PLPKI in different clones of *Solanum tarijense*, a species of potato that functions as a natural reservoir of genes controlling resistance against diseases and pests (Buso et al. 2003).

Materials and Methods

Plant growth and fungal culture propagation: Potato cultivars and fungal microorganism cultures were provided by the Balcarce Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina. Potato plants with different degrees of horizontal resistance such as *Solanum tuberosum* L. cv. 'Pampeana' INTA (moderately resistant); 'Bintje' (susceptible); *Solanum tarijense* clones (*trj*) and OKA5632.12 (moderately resistant); OKA 5632.11 (resistant); and 'HOF 1717.10' (susceptible) were grown as previously described (Feldman et al. 2000). The area under the disease progress curve (AUDPC) data for each clone were determined by Capezio et al. (2008), as expression of the behaviour of the plant in relation to the pathogen (Shanner and Finney 1977).

HOF 1717.10 scored the highest AUDPC values, whereas OKA5632.11 showed the best performance against *P. infestans* and the lowest AUDPC value (Table 1).

Phytophthora infestans (races 1, 4, 7, 8, 10, 11, mating type A2) was cultured on modified Plitch liquid medium containing 0.1 mg/ml of denatured potato protein. *R. solani* anastomosis group AG3 (*Rs*AG3) and binucleate *Rhizoctonia* N2 (*R*N2) were grown on Maxwell liquid medium (Maxwell and Lumsden 1970) containing 0.1 mg/ml of denatured potato protein. *P. infestans* and *Rhizoctonia* spp. were cultivated for 7 days at 18°C. Cultures were filtered, dried at 60°C for 12–15 h and weighed for fungal biomass determinations. Cleared culture medium was used as source of extracellular microbial proteases.

Protease and protease inhibitor assays: Extracellular fungal and oomycete proteolytic activities were determined with azocasein as substrate in 50 mM Tris–HCl (pH 8.0), 1 mM $CaCl_2$ and suitable amounts of culture filtrates. Samples were maintained at 37°C for 2 h. Protease activity was monitored as an increase in the absorbance (335 nm) of the supernatant.

The effect of protease inhibitors was tested as described above, with the addition of inhibitors at the following final concentrations: 0.02 mM aprotinin, 0.05 mM pepstatin A, 0.62 mM E-64, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM and 80 mM EDTA, 5 µM soybean trypsin inhibitor and different amounts of PLPKI. Commercial inhibitors were purchased from Sigma (USA), while PLPKI was purified from potato leaves as previously described (Feldman et al. 2000).

The ability of a chemical/PLPKI to inhibit extracellular proteases was determined by comparing the proteolytic activity of the culture filtrate in the presence or absence of an inhibitor. This value was used to calculate the percentage of proteolytic activity inhibited. The experiment was repeated twice with 3 replicas per determination. Protein concentration was determined by the Bradford method (Bradford 1976).

NH$_2$-terminus sequence determination of PLPKI: The purified PLPKI (Feldman et al. 2000) was fractionated on a 18% polyacrylamide gel according to the method of Laemmli (1970). The protein was electrophoretically transferred to Problott™ PVDF membrane (Applied Biosystems, USA), using an electrophoretic transfer cell (Trans-Blot, Bio-Rad, USA). The membrane-immobilized PLPKI was submitted to the Protein Sequencing Facility at the University of California at

Riverside. The NH$_2$-terminal sequence of purified PLPKI was determined by automated Edman degradation performed with a liquid-phase sequence analyser (Model 492; Applied Biosystems, USA). The peptide sequence was submitted to the Swiss Protein Sequence Bank to determine its homology to plant protease inhibitors.

Protein extraction and immunoblot analysis: Leaves from each clone/cultivar were homogenized in 50 mM Tris–HCl, pH 7.5, containing 0.1% (v/v) β-mercaptoethanol, 0.6 M NaCl and 0.133% (v/v) Tween-20. After centrifugation, the supernatant was dialysed against 50 mM Tris–HCl, pH 8.0, and submitted to thermal treatment at 80°C for 10 min.

For immunoblots, protein extracts (6 mg FW/lane) were fractionated on a 18% SDS–polyacrylamide gel and transferred onto nitrocellulose using a semi-dry electrophoretic transfer cell (Trans-Blot, Bio-Rad, USA). Immunodetection was performed as described by Turner (1986) using a polyclonal antibody raised against PLPKI (Feldman et al. 2000) and visualized with a chemiluminescence kit (Pierce). PLPKI accumulation was quantified with Scion Image software (Scion Corporation).

Results

PLPKI: a new member of the protease inhibitor I family

The first 20 residues (LFCQKGLRWPELIGVPAYQA) at the N-terminus of the mature PLPKI, purified from *Pi*-infected potato leaves, were determined. Comparison of the N-terminal sequence of PLPKI to polypeptides in the Swiss-Prot databank showed that the N-terminus of PLPKI had a high degree of sequence similarity to the potato protease inhibitor I family (Table 2). A conserved 8-residue motif (WPELIGVP) corresponding to the 9 to 16 PLPK1 residues was found near the N-terminus of ten different potatoes, two tomatoes and two tobacco protease I inhibitors. Only five of these eight residues were conserved in the non-solanaceous *Cucurbita maxima* protease inhibitor I family (Table 2).

When residues adjacent to the 8-residue conserved motif were examined, it was found that they were distinct from all 18 protease inhibitors that were compared, with the exception of Ala-20 and Cys-3 (Table 2). In the N-terminal region, PLPKI was more divergent from other potato protease inhibitors than they were from each other (Fig. 1).

Extracellular proteases secreted by *P. infestans* and two *Rhizoctonia* species

The activities of extracellular proteases secreted by *Pi*, *Rs*AG3 and binucleate *R*N2 were determined after seven days of growth in liquid media containing denatured potato proteins as carbon source. Total proteolytic activity in the culture filtrates, expressed as U/ml or U/g, was similar for all three organisms (Fig. 2a, b).

To determine the classes of proteases secreted into the media, their sensitivities to six protease inhibitors, PMSF, EDTA, pepstatin A, E-64, aprotinin and soybean trypsin inhibitor, were determined (Table 3). Although their total proteolytic activities were similar (Fig. 2), each microorganism secreted a different array of enzymes. The proteolytic activity from culture filtrates of *Pi* was partially eliminated by PMSF (14%) and EDTA (75%). However, proteases secreted by *Pi* were not inhibited by pepstatin A, E-64, aprotinin or soybean trypsin inhibitor. These data indicate that the proteases secreted by *Pi* were primarily serine and metalloproteases.

Analysis of culture filtrates of *Rs*AG3 showed that, like *Pi*, most proteolytic activity was associated with serine and

Table 1: Relative area under the disease progress curve (AUDPC), in potato clones of *S. tarijense* (*trj*) and *S. tuberosum* (*tbr*) cultivars (Capezio et al. 2008)

tjr or *tbr* Genotype	AUDPC (area under the disease progress curve)
OKA 5632.11	94
OKA 5632.12	257
Pampeana	272
Bintje	310
HOF 1717.10	335

Table 2: Comparison of the NH$_2$-terminal amino acid sequence of PLPKI with plant protease inhibitors of the potato family I

Species/Enzyme	Peptide Sequence[A]	Accession Number
potato PLPKI	LFCQKG---LRWPELIGVPAQYA	This paper
potato PinI	feC-nG--kqRWPELIGVPAQYA	AAZ08249.1
potato PinI	fqC-nG--kLsWPELIGVPtklA	AAS46024.1
potato PinI	fqC-KG--kLRWPELIGVPtklA	Q00783
potato PinI	feC-nG--kqRWPELIGVPtklA	P08454.2
potato PinI	feC-nG--keRWPELIGVPtklA	1314299A
potato PinI	feC-nG--kqfWPELIGVPtklA	AAA69780.1
potato PinI	feC-nG--kLsWPELIGVPAhYA	AAC49603.1
potato PinI	feC-dG--kLqWPELIGVPtklA	P01052.1
potato PinI	feC-KG--kLqWPELIGVPtklA	AAZ08245.1
potato PinI	feC-nG--kLqWPELIGVPtklA	S27218
tomato PinI	LmC-eG--kqmWPELIGVPtklA	P05118.1
tomato PinI	sFC-pGvtkesWPELlGtPAkfA	NP_001234615.1
L. peruvianum PinI	srC-KG--kqfWPELIGVPAlYA	AAA34198.1
N. tabacum PinI	sgC-pGvtkeRWPELlGtPAkfA	Q03199.1
N. sylvestris PinI	--------ketWPELIGVPAkfA	Q02214.1
N. glauca X N. langsdorffii PinI	--------ketWPELIGVPAkfA	BAA05472.1
N. glauca X N. langsdorffii PinI	--------ketWPELlGVPAkfA	BAA02823.1
Cucurbita maxima PinI	--C-pG--kssWPhLvGVggscA	P19873

Polypeptide sequences were aligned and gaps (indicated as ashes) were introduced to achieve best identities. The highly conserved 8-residue motif and the conserved Ala residue are highlighted. PLPKI sequence was determined by Edman degradation (see *Materials and Methods*). The tomato, potato, tobacco and cucurbit protease inhibitor sequences can be retrieved from the sequence accessions indicated.

metalloproteases. However, the balance of serine and metalloproteases was distinct in this fungus. The majority of proteolytic activity was attributed to serine proteases, as PMSF inhibited 67% of activity in culture filtrates. In contrast, no serine protease activity was detected in the culture filtrates of the binucleate *R*N2. Instead, pepstatin A and EDTA inhibited about 7% and 80% of the total proteolytic activity, respectively, indicating the presence of aspartic and metalloproteases in the culture medium. The fact that none of the extracellular proteolytic activities were inhibited by E-64 suggests the absence of cysteine proteases in the culture media.

PLPKI inhibited proteases secreted by *P. infestans* and *Rhizoctonia spp.*

To determine the potential role of PLPKI in defence responses against pathogens, inhibition of the proteases secreted by *Pi*, *Rs*AG3 and binucleate *R*N2 was assessed. Figure 3 shows that PLPKI inhibited 13% and 30% of the secreted proteolytic activities produced by *Pi* and *Rs*AG3, respectively. Reciprocal plots of data obtained with higher concentrations of PLPKI (up to 5 µg) showed that increases in PLPKI did not cause further increments in the levels of inhibition (not shown). On the other hand, PLPKI did not influence the proteolytic activity in culture filtrates of the binucleate *R*N2 (Fig. 3).

PLPKI accumulation and activity in clones with different degrees of horizontal resistance to *P. infestans*

To determine whether PLPKI content was correlated with the degree of horizontal resistance to *Pi*, we analysed its constitutive expression in five potato cultivars with different degrees of resistance by Western blot. Clone OKA 5632.11, the most resistant cultivar, showed the highest level of PLPKI accumulation, 14 times more than 'HOF 1717.10', the most susceptible cultivar (Fig. 4). When total proteinase K inhibitory activity was quantified, the highest activity was detected in the same clone (80% of protease K inhibitor activity). However, clone OKA 5632.12, moderately resistant, also showed high inhibitory activity. This could be explained by the fact that total protease inhibitor activity was analysed. In the rest of the clones, which showed a higher AUDPC (more susceptible), the percentage of inhibitory activity was lower (20–30%) (Fig. 5).

Discussion

The potato protease inhibitor I family is a widely distributed family among the serine PIs known in plants (Habib and Fazili 2007). A subset of the genes in the protease inhibitor I family are induced in pathogen-infected, insect-infected or wounded plants (Geoffroy et al. 1990, Pautot et al. 1991, Cordero et al. 1994, Tamayo et al.
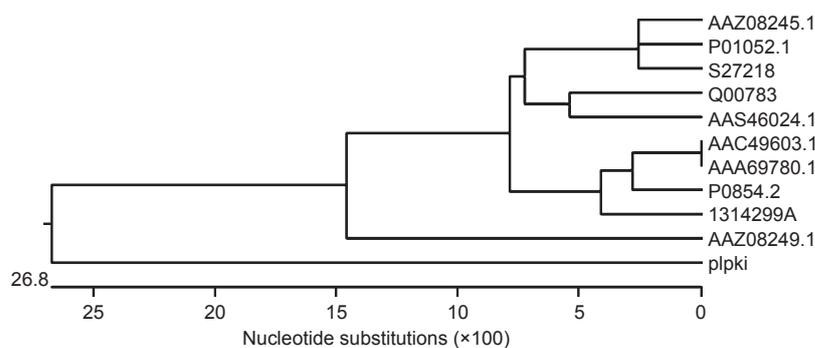
Fig. 1: Nucleotide divergence between potato I protease inhibitors and PLPKI
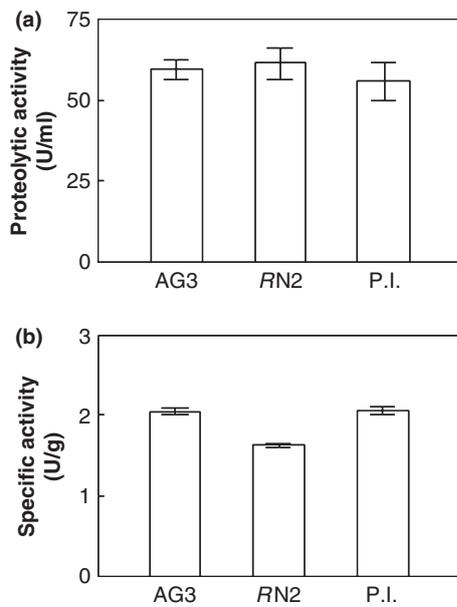
Fig. 2: Extracellular protease production by *P. infestans*, *R. solani* AG-3 and binucleate *Rhizoctonia* N2 (BNR N2). (a) Total protease activity, U/ml, of filtrate culture. One proteolytic unit (U) was defined as an increase in absorbance at 335 nm of 0.1 in 1 h at 37°C. (b) Specific proteolytic activity, U/g microorganism dry weight. Values are means (± SD) from three separate determinations (the experiment was repeated twice)

Table 3: Inhibition of proteolytic activity from filtrate cultures of *P. infestans*, *R. solani* AG3 and binucleate *Rhizoctonia* N2 by protease inhibitors

Inhibitor	Inhibition of secreted proteases (%)[1]		
	P. infestans	*R. solani* AG3	BNR N2
PMSF	14.4 ± 1.91	66.87 ± 4.33	0
EDTA 1 mM	46 ± 2.24	20.77 ± 3.84	81.12 ± 4.3
EDTA 80 mM	74.7 ± 6	0	0
Pepstatin A	0	0	6.57 ± 0.89
E-64	0	0	0
Aprotinin	0	0	0
Soybean Trypsin Inhibitor	1.76 ± 0.3	0	0

[1]Culture filtrates from *P. infestans*, *R. solani* AG3 and binucleate *Rhizoctonia* N2 (BNR N2) were isolated. Proteolytic activity and the impact of protease inhibitory compounds were determined. Data are expressed as % means ± SD of three different experiments.

2000, Turrá et al. 2009, Turrá and Lorito 2011). A small number of these PIs are active against proteases of microbial origin (Geoffroy et al. 1990, Hermosa et al. 2006, Ievleva et al. 2006), and among them, the potato PLPKI, a serine PI that inhibits the proteolytic activity of the microbial enzymes proteinase K and subtilisin, was purified (Feldman et al. 2000). In that work, it was reported that high levels of PLPKI were detected after *Pi* infection in a potato cultivar ('Pampeana' INTA) described to have a high degree of horizontal resistance, whereas this protein was undetectable in an infected susceptible cultivar ('Bintje').

To compare PLPKI with other PIs from potato and other plants, the NH$_2$-terminal sequence of the purified, mature PLPKI was determined. The peptide sequence showed that PLPKI was a member of the potato protease inhibitor I family (Table 2). A highly conserved eight-residue motif (PLPKI amino acid residues 9–16) and invariant Ala-20 and Cys-3 were found in all the potato and several of the tomato and tobacco PIs belonging to
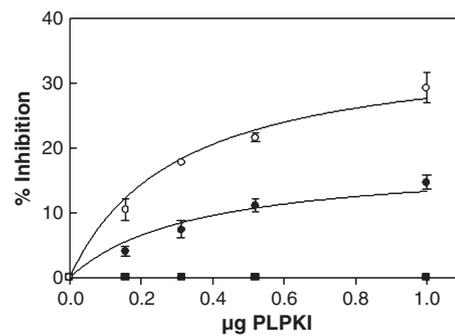
Fig. 3: Inhibitory activity of PLPKI against proteases secreted by *P. infestans*, *R. solani* AG3 and the binucleate *Rhizoctonia* N2. In all assays, control extracts (100% activity) had the same activity (10 U/h). PLPKI activity was expressed as the% of the control proteolytic activity that was inhibited. (○, *R. solani* AG3); (●, *P. infestans*); (■, binucleate *Rhizoctonia* N2). Values are means (±SD) from three separate determinations (the experiment was repeated twice)
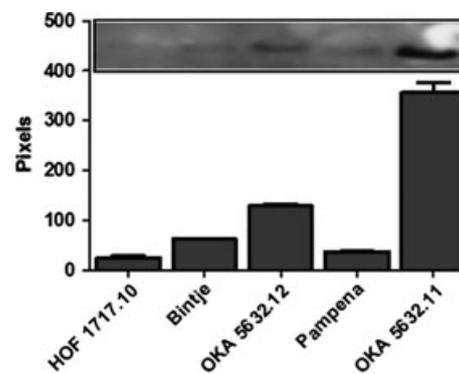
Fig. 4: PLPKI levels in clones with different degrees of horizontal resistance. Protein extracts (6 mg FW/lane) were fractionated on an 18% SDS–polyacrylamide gel and transferred onto nitrocellulose. Immunodetection was performed using polyclonal antibodies raised against PLPKI. Values are means (±SD) from three separate determinations with Scion Image software
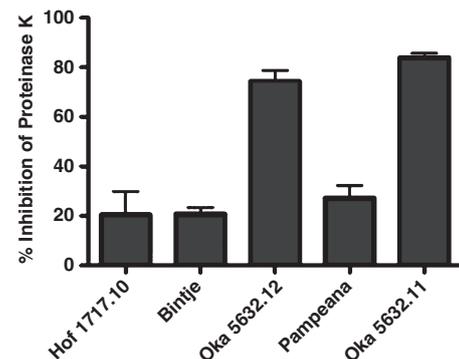
Fig. 5: Proteinase K inhibitor activity in clones with different degrees of horizontal resistance

the potato I family. Similar to other members of the potato protease inhibitor I family, PLPKI has an oligomeric structure (Feldman et al. 2000).

As the N-terminal sequence of PLPKI was distinct from the other potato protease inhibitor sequences in protein/gene sequence databanks (Table 2) and showed to be more divergent from other potato PIs than they were from each other (Fig. 1),

PLPKI appears to be a novel member of this family of protease inhibitors.

The production of extracellular proteases by plant pathogenic fungi is well documented, and it has been proposed that, in some fungal–plant interactions, these enzymes may function as pathogenicity factors (Ryan 1973, Ball et al. 1991, Dobinson et al. 1997, Knogge 1998, París and Lamattina 1999, Gvozdeva et al. 2004, 2006, Valueva and Mosolov 2004, Ievleva et al. 2006). In the present work, we partially characterized extracellular protease activities secreted by two phytopathogens, the oomycete *P. infestans* and the fungus *R. solani* AG3, and one non-pathogenic fungus, *Rhizoctonia* N2. Using a variety of protease inhibitors, it is shown that each pathogen secreted a different array of proteolytic activities. The culture media of *Pi* secreted mainly serine and metalloproteases being the latter more abundant. Similar results were described by Gvozdeva et al. (2004). In the case of *Rs*AG3, this ratio was the opposite. *Pi* metalloproteases were similar to enzymes in the alkaline protease group that require more than 50 mM EDTA to completely inhibit the metalloprotease activity (Sexton et al. 1994). This is in sharp contrast with *Rs*AG3 and *R*N2 metalloproteases, which were inhibited by 1 mM EDTA. Despite the fact that the proteolytic activities of *Pi* and *Rs* culture filtrates were partially inhibited by PMSF, there was no inhibition by aprotinin or soybean trypsin inhibitor, two serine PIs with high specificity against trypsin-like proteases. On the other hand, PMSF reacts only slightly with trypsin (Powers and Harper 1986), and PLPKI has the highest inhibitory activities on serine proteases of microbial origin (i.e. subtilisin) (Feldman et al. 2000). These data suggest that the serine protease activities of *Pi* and *Rs* could be, at least in part, of the subtilisin-like type.

It has been shown that some plant protease inhibitors have activity against proteases from microbial sources (Mosolov et al. 1979, Geoffroy et al. 1990, Ryan 1990, Marchetti et al. 1995, Gozia et al. 1996, Feldman et al. 2000, Gvozdeva et al. 2004, 2006, Hermosa et al. 2006). In the present study, evidence that showed that PLPKI inhibited the activity of extracellular proteases produced by two phytopathogens was presented. While PLPKI (1 μg) reduced the activity of extracellular proteases from *Pi* and *Rs*AG3 cultures, this inhibitor had little effect on proteases produced by the binucleate *R*N2. Binucleate *R*N2 isolates are non-pathogenic on potato, and infection by this fungus confers an induced resistance to *Rs*, the causal agent of Rhizoctonia canker (Escande and Echandi 1991a,b). PLPKI appears to be a very effective inhibitor of serine proteases secreted by *Pi* as serine proteases represented 14% of the *Pi* culture fluid (PMSF-sensitive proteases) and PLPKI inhibited 13% of the total protease activity in these preparations. In contrast, PLPKI was less effective against serine proteases produced in *Rs*, as this protein inhibited only 43% of the serine protease activity in *Rs* cultures. The ability of PLPKI to completely inhibit serine protease activities from *Pi* suggests a kind of specificity of this inhibitor in the *Pi*–potato interaction. Interestingly, in the binucleate *R*N2 culture media, the main proteolytic activities corresponded to metallo- (81%) and aspartic (7%) proteases (Table 3). These results could explain the fact that PLPKI did not affect the proteolytic activities of this fungus.

It is clear from recent studies on microbial phytopathogens that the importance of proteases in the disease process depends upon the specific interaction (Robertsen 1984, Ball et al. 1991, París and Lamattina 1999). Although in this work it has been demonstrated that PLPKI has inhibitory activity against secreted proteases from pathogenic microorganisms, the involvement of *Pi* and *Rs* proteases in the growth of these pathogens *in planta* and in the development of disease symptoms remains to be determined. Further studies on pathogen proteases and PLPKI will allow dissecting their biological roles in the potato–*Pi* interaction.

Phytophthora infestans is the causal agent of late blight. Traditional control of this disease is made by frequent agrochemical applications; however, this strategy is expensive and harmful for the environment. In this scenario, horizontal resistance is recognized as the potentially most effective and environmentally acceptable method for late blight management. In Argentina, several sources of horizontal resistance were identified. In particular, the wild species *Solanum tarijense* (*trj*) has shown high levels of resistance. Previous results with *trj* clones resistant to late blight showed an induction of pathogenesis-related protein activities (Wolski et al. 2009, Korgan et al. 2010). In this study, three *S. tarijense* clones with known horizontal resistance to *Pi* were used to determine whether there was a correlation between *S. tarijense*-derived resistance to *Pi* and PLPKI content. Our results showed a clear correlation between PLPKI accumulation/activity and the degree of horizontal resistance. PLPKI accumulated to high levels in OKA 5632.11, a clone described as highly resistant (Capezio et al. 2008, Mugica et al. 2010). Although future analysis of a higher number of clones will shed light into the extent of this correlation, the fact that PLPKI is constitutively expressed in clones with different degrees of horizontal resistance suggests that PLPKI might be a suitable biochemical marker to assist breeders in the selection of HR cultivars.

To understand the specificity of PLPKI and the mechanisms that regulate PLPKI expression in *Pi*-resistant and -susceptible cultivars, cloning and characterization of the *plpki* gene are in progress.

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