Research article

Hydrophobic proteins secreted into the apoplast may contribute to resistance against *Phytophthora infestans* in potato

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ABSTRACT

During plant–pathogen interaction, oomycetes secrete effectors into the plant apoplast where they interact with host resistance proteins, which are accumulated after wounding or infection. Previous studies showed that the expression profile of pathogenesis related proteins is proportional to the resistance of different cultivars toward *Phytophthora infestans* infection. The aim of this work was to analyze the expression pattern of apoplastic hydrophobic proteins (AHPs), after 24 h of wounding or infection, in tubers from two potato cultivars with different resistance to *P. infestans*, Spunta (susceptible) and Innovator (resistant). Intercellular washing fluid (IWF) was extracted from tubers and chromatographed into a PepRPC™ HR5-5 column in FPLC eluted with a linear gradient of 75% acetonitrile. Then, AHPs were analyzed by SDS-PAGE and identified by MALDI-TOF-MS. Innovator cv. showed a higher basal AHP content compared to Spunta cv. In the latter, infection induced accumulation of patatins and protease inhibitors (PIs), whereas in Innovator cv. no changes in PIs accumulation were observed. In response to *P. infestans* infection, lipoxygenase, enolase, annexin p34 and glutarredoxin/cyclophilin were accumulated in both cultivars. These results suggest that the AHPs content may be related to the protection against the oomycete and with the degree of potato resistance to pathogens. Additionally, a considerable number of the proteins putatively identified lacked the signal peptide and, being SecretomeP positive, suggest unconventional protein secretion.

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1. Introduction

The apoplast is the portion outside the cell membrane that includes cell walls and intercellular space of plants [1]. In this compartment, important events take place to regulate plant physiological and developmental processes [2]. The intercellular space is a dynamic environment that represents the outermost barrier of the cell; it also plays an important role in plant resistance to biotic and abiotic stresses. Considering that invasion of the plant apoplast is a critical phase of the infection cycle of pathogens, it can be postulated that this compartment serves as a molecular battlefield that contributes either to the success of pathogen infection or to plant resistance [3]. In response to pathogen attack, plant cells secrete a wide range of defense-related proteins into the apoplast, including antimicrobial peptides, detoxifying enzymes and pathogenesis-related proteins (PRs) whose function still remains unknown [4–6]. Many of these PRs have antimicrobial activity and hydrophobic characteristics [4,7–10].

Phytophthora infestans is the causal agent of late blight, a reemerging and ravaging disease of potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) [11–14] that generates losses over 70% of the annual potato crop yields. *P. infestans* is thought to accomplish parasitic colonization by molecular reprogramming of host defense circuitry, specifically by introducing an array of effectors that function in the plant apoplast and cytoplasm [15]. Two forms of genetic resistance to *P. infestans* in potato species have been described: race-specific (vertical resistance) or race non-specific (horizontal or field resistance) [16]. Race specific resistance is characterized by interactions between products of dominant R genes in the host and corresponding avirulence (avr) genes in the pathogen. In contrast, race-non-specific resistance or field resistance is assumed to be multiple genes based. This type of resistance is durable and thus commercially more attractive than race-specific resistance [17–23].

Proteomics is a potential tool for dissecting molecular mechanisms underlying plant–pathogen interactions. Proteomic changes in the host plant during pathogen infection are of great interest, because they may give insight into critical 'switch points' in the defense-related pathways that could be manipulated to engineer host plants with improved resistance or immunity to the pathogen. Based on the antecedents above described, the aim of this work was to analyze if apoplastic hydrophobic proteins (AHP) content could be correlated with the potato field resistance to *P. infestans*. Additionally, analysis by FPLC, SDS-PAGE and mass spectroscopy (MALDI-TOF-MS) were performed to identify apoplastic hydrophobic proteins differentially expressed in healthy or infected tubers of two potato cultivars with different field resistance degree to *P. infestans*.

2. Results

2.1. Isolation of secreted proteins from healthy, wounded and infected tubers

Intercellular washing fluids (IWFs) from healthy, wounded and *P. infestans* infected tubers were obtained using a protocol based on infiltration-centrifugation [24]. Possible contamination of IWFs with intracellular components was tested by measuring activities of α-mannosidase and glucose-6-P-dehydrogenase, as markers for vacuoles and cytoplasm, respectively. Less than 1% of total marker enzyme activity was found in the IWFs of healthy or infected tubers.

To isolate apoplastic proteins with hydrophobic characteristics (AHPs), IWFs of control, wounded and *P. infestans* infected tubers were fractionated by high-resolution liquid chromatography (FPLC) using a hydrophobic interaction column (PepRPC™ HR5/5 column) and eluted with 75% (v/v) acetonitrile, as described in Materials and Methods. Subsequently, to perform the quantitative analysis of the isolated AHPs, protein concentration was measured in all of eluted fractions. Results show that (Fig. 1) there are basal differences in de AHPs content between cultivars. Healthy tubers of the resistant cultivar (Innovator cv.) contained twice as much AHPs than healthy tubers of susceptible cultivar (Spunta cv.). This value was expressed in Fig. 1 as a ratio AHP content/apoplastic total proteins (ATP) content, since there are no differences between the
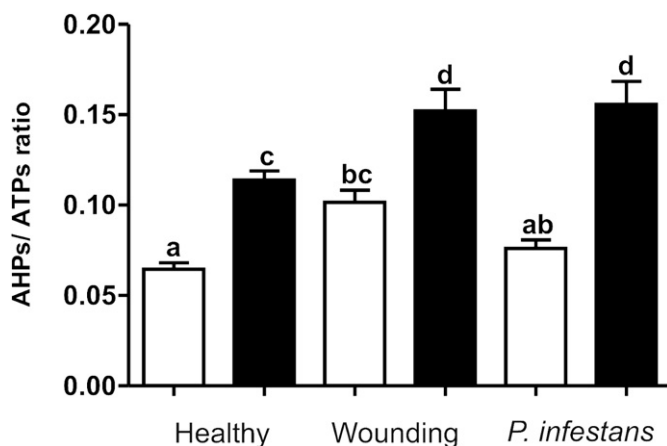
ATPs content present in the IWFs of different cultivars or tuber treatment. In cv. Innovator, AHPs content was 0.5 fold higher in wounded and infected tubers compared the healthy ones. However, an increase in the AHPs content was observed in wounded tubers respect to healthy tubers of the susceptible cultivar.

2.2. Identification and analysis of hydrophobic secreted proteins

In order to analyze the AHPs pattern in IWFs corresponding to different treatments, SDS-PAGE analysis was performed (Fig. 2). Thereafter, differential bands were recovered, digested with trypsin and submitted to a MALDI-TOF spectrometric analysis in order to identify the proteins. The obtained peptide mass fingerprints were used to search the National Center for Biotechnology Information database using MASCOT (http://www.matrixscience.com) software, BLASTp and the NCBInr database. Sequence coverage of 20%, a score of 59 and a peptide number of 4 were established as cut off of tentative identification. Using this strategy, 15 proteins were putatively identified and the information obtained is summarized in Table 1, where it can be seen that several bands matched with proteins of *S. tuberosum* and other matched belonging to plant genera different from *Solanum* and are still unknown potato proteins.

Table 1 and Fig. 3 show that, most of proteins of SDS-PAGE (66.7%) were identified as protease inhibitors (PIs). Proteins of the band 5 matched with a cathepsin D inhibitor from *Solanum nigrum* (GenBank ID: AAG12337); proteins corresponding to the band 6 matched with the sequence of a cysteine protease inhibitor from *S. tuberosum* (GenBank ID: AAB28594 (**PCPI 8.3**)); proteins corresponding to the bands 9 and 10 matched with the aspartic protease inhibitors from *S. tuberosum* (GenBank IDs: P58518 (**API-3**) and P16348 (**PDI**)); and finally, proteins corresponding to the bands 7, 8, 11, 12, 13 and 15 matched with Kunitz type protease inhibitors from *S. tuberosum* (GenBank IDs: ABA81855; AAM10743 (**P1H5**); AAM21644 (**P2G2**); AAU09271 (**PKPI-B2**); P58515 (**PSPI**) and AAB32802 (**PKPI-20**)).

Fig. 1. AHPs/ATPs ratio in tuber intercellular washing fluids. Apoplastic hydrophobic and total proteins were isolated from two cultivars with different field resistance to *P. infestans* after 24 h of infection: Spunta (susceptible, white bars) and Innovator (resistant, black bars). Results are expressed as the AHPs/ATPs ratio and represent means ± SEM of triplicate samples. Different letters indicate significant differences with $p < 0.05$ for One-Way ANOVA and Tukey tests.

Fig. 2. Analysis of apoplastic hydrophobic proteins of potato stressed tubers by SDS-PAGE. The equivalent to 75 mg of fresh weight of apoplastic hydrophobic proteins obtained by HIC (elution at 75% acetonitrile) were separated on 15% SDS-PAGE gel and silver nitrate stained. Lane 1, control ($t = 0$); lane 2, wounding; lane 3, *P. infestans* infection. Numbers indicate protein identification by MALDI-TOF-MS. These gels are representative of three independent experiments of three sets of samples.

Table 1
Putative apoplastic hydrophobic proteins (AHPs) differentially expressed in potato tubers after *P. infestans* infection.

Band	MALDI-TOF-MS protein identification	Class	Accession number	Score	Coverage (%)	MW kDa exp/theor	SignalP	SecretomeP
1	Enolase (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase)[a]	II	P26300	171	20	77.62/97.178	No	Yes
2	Patatin-07	I	ABC55694	184	21	48.91/42.73	Yes	No
3	Patatin-13	I	ABC55700	151	48	46.23/42.45	Yes	No
4	Annexin p34-like protein-like	III	ABB55363	65	22	40.83/36.023	No	No
5	Cathepsin D inhibitor[b]	NA	AAG12337	138	54	23.28/20.086	NA	NA
6	Cysteine protease inhibitor	II	AAB28594	72	30	32.96/20.305	No	Yes
7	Kunitz type protease inhibitor	I	ABA81855	89	51	21.97/24.862	Yes	No
8	Putative kunitz-type proteinase inhibitor precursor P1H5	I	AAM10743	263	62	19.63/24.494	Yes	No
9	Aspartic protease inhibitor 3	NA	P58518	93	32	20.180/18.909	NA	NA
10	Aspartic protease inhibitor 11	NA	P16348	92	29	19.91/20.919	NA	NA
11	Putative Kunitz-type proteinase inhibitor	I	AAM21644	114	38	18.96/20.491	Yes	No
12	Kunitz-type proteinase inhibitor group B	NA	AAU09271	313	57	17.06/21.248	NA	NA
12	Serine protease inhibitor 2	III	P58515	89	38	14.39/20.331	No	No
13	Putative Kunitz type protease inhibitor	I	AAM21644	89	38	14.39/20.491	Yes	No
14	Glutaredoxin	III	ABU96710	58	22	13.12/11.428	No	No
	Cyclophilin[c]	III	AAD22975	59	23	13.12/18.199	No	No
15	Kunitz type protease inhibitor (N-terminal)	NA	AAB32802	82	75	11.96/2.344	NA	NA

Band: Band identified on the gel in Fig. 2; MW kDa exp/theor: MW kDa experimental/theoretical; NA: not analyzed, mature proteins.
The analysis with SignalP software was used to detect the N-terminal signal peptides signature (Class I), and SecretomeP for pathway-independent features of mammalian secreted proteins (Class II). Class III includes proteins resulting SignalP and SecretomeP negative.
[a] From *Solanum lycopersicum*.
[b] From *Solanum nigrum*.
[c] From *Solanum tuberosum subsp. tuberosum*.

The remaining proteins (28.5%) matched with: 1 – an enolase from *S. lycopersicum* (GenBank ID: P26300), corresponding to band 1 of the SDS-PAGE; 2 – a glutaredoxin/cyclophilin (GenBank ID: AAD22975 and ABU96710, correspond to band 14 SDS-PAGE band). 3 – two bands (corresponding to 2 and 3 of the SDS-PAGE) matched with patatins *S. tuberosum*, patatin-07 (GenBank ID: ABC55694) and patatin-13 (GenBank ID: ABC55700); 4 – an annexin-like protein from *S. tuberosum* (GenBank ID: ABB55363) corresponding to band 4 of the SDS-PAGE. Only 4.8% of the proteins present in the isolated bands were not identified.

Densitometric analysis of SDS-PAGE bands shows that the amount of most PIs identified was higher in healthy tubers of resistant cultivar (Innovator cv.) than in healthy tubers of susceptible one (Spunta cv.) (Fig. 4). However, no change was recorded in the amount of PKPI-B2 (AAU09271) and PKPI-20 (AAB32802) after *P. infestans* infection in the resistant cultivar. On the other hand, protein bands that matched with patatin-07 and -13 were visualized in the apoplast of tubers of both potato cultivars after *P. infestans* infection. However, only in the resistant cultivar these protein bands were observed in the apoplast of healthy tubers. SDS-PAGE protein bands that matched with annexin-like protein, enolase form *S. lycopersicum*, glutaredoxin and cyclophilin were only visualized after *P. infestans* infection.
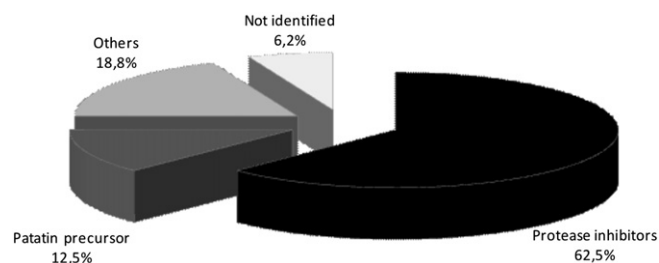

Fig. 3. Classification of hydrophobic secreted proteins from 24 h infected tuber. Protein classification from MALDI-TOF-MS identified proteins in 24 h infected IWFs was performed. (A) Protein group identification (B) protease inhibitors families. These values were obtained from three independent experiments of three samples.

3. Discussion

3.1. Correlation between apoplastic hydrophobic protein content and the degree of potato field resistance to P. infestans

In this work we have used proteomics to decipher molecular processes that occur during the recognition of pathogens by plants. Specifically, we analyzed the possible correlation between the hydrophobic protein content in potato tuber apoplast and the degree of field resistance of potato to *P. infestans*. Additionally, we have analyzed the effect of wounding on tuber AHPs accumulation, since previous reports on inoculated tubers suggest that intact tuber periderm provides a barrier to *P. infestans* penetration [34–36] and infections develop only through wounds, eyes and lenticels (in order of preference) [34–38].

Results obtained here suggest that there are constitutive differences that would connect the AHPs level with a low or high potato defense response to *P. infestans* attack. This is based on the fact that there is a higher amount of AHPs in healthy tubers of resistant cultivar than in the susceptible one. This result is in accordance with the reported by Vleeshouwers et al. [39], who demonstrated that constitutive expression of PR genes may contribute to non-specific resistance to *P. infestans* in *Solanum*. Results obtained in this work and the antecedents above described suggest that the identification of mRNAs from PRs involved in the partial resistance of Solanaceae to economically important oomycetes, such as *P. infestans*, is an interesting starting point to generate new tools for the managing of the breeding programs.

3.2. Proteomic analysis of differential secreted hydrophobic potato proteins

Most of the SDS-PAGE bands corresponding to AHPs induced after wounding or infection in both cultivars matched with protease inhibitors: the cysteine protease inhibitor, **PCPI 8.3**, the aspartic protease inhibitors **API-3** and **PDI**, the Cathepsin D inhibitor, **CDI** and the Kunitz type protease inhibitors: **P1H5**, **P2G2**, **PSPI** and **PKPI-20**. These inhibitors may play a significant role in the
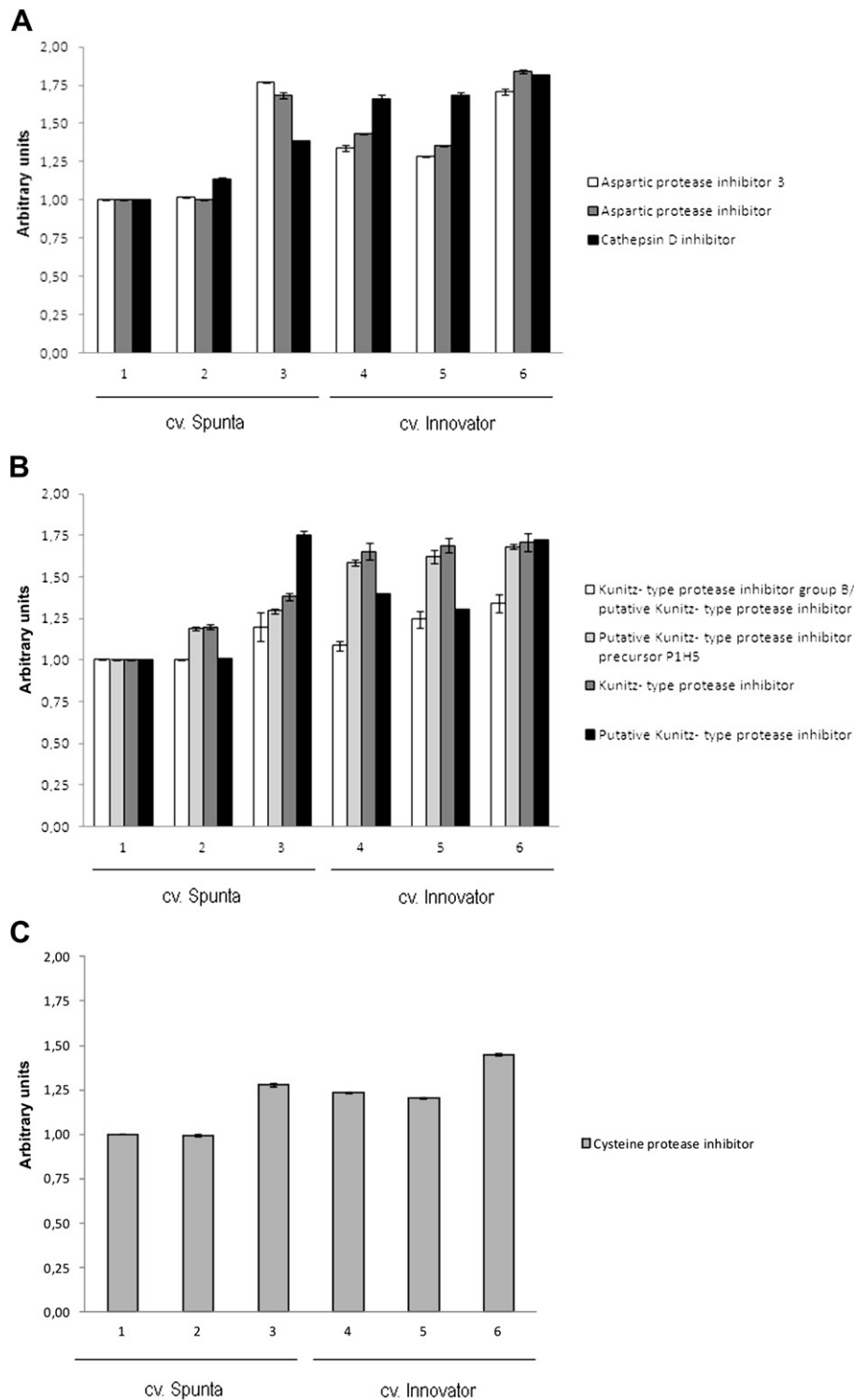
Fig. 4. Densitometric analysis of differentially expressed tuber apoplastic protease inhibitors after 24 h of wounding or *P. infestans* infection. Band intensities of apoplastic hydrophobic aspartic (A), serine (B) or cysteine (C) protease inhibitors were quantified by densitometric analysis using the Image J software. Results are expressed as arbitrary units, considering cv. Spunta control as one. Lane 1: control ($t = 0$); lane 2: 24 h wounding; lane 3: 24 h *P. infestans* infection. Results are expressed as arbitrary units taking cv. Spunta control condition as one. Bars represent means ± SEM, these values were obtained from three independent experiments of three sets of samples.

natural defense mechanisms of the potato plant against insect and phytopathogen attack and have a high toxicity toward the pathogen, inhibiting the germination of hyphae and accelerating the destruction of fungal spores [40–42]. The induction by wounding and infection of these proteins can be explained since there are precedents for cross-talk between both pathways, where many of the same genes and enzymes can be induced by both, pathogens and wounding [43,44]. Previously, the induction of these proteins in abiotic or biotic stress conditions has been described. Valueva et al. [45] reported that in the susceptible cultivar the proteins that

matched with PSPI are induced after *P. infestans* infection. Mares et al. [46] described the potato cathepsin D inhibitor (CDI), that was wound-inducible in potato leaves, both locally and systemically [47]. CDI can be detected in tuber, stem and root tissue [48]. Also, Hansen and Hannapel [49] showed that heterologous CDI genes were wound-inducible for all Solanaceae species assayed. However, the novelty of this work is to show that higher basal amounts of this inhibitor in potato tubers may contribute to non-specific resistance to *P. infestans* in *Solanum*. Additionally, the higher amount of Kunitz type protease inhibitors in resistant cultivar and their induction after *P. infestans* infection observed here reinforce the results obtained by Valueva et al. [40] that shows that the induction of this proteins contribute to potato resistance, since PKPIs inhibit the growth of hyphae of *P. infestans* and damage zoospores of this pathogen in a dose dependent manner.

As well as most of AHPs that matched with PIs, proteins present in two SDS-bands that matched with patatins were only detected in the apoplast of healthy tubers from resistant cultivars and in the apoplast of infected tubers of both cultivars. Patatins are non-specific lipid acyl hydrolases that account for approximately 40% of the total soluble protein in mature potato tubers, and it has potent insecticidal activity against the corn rootworm. All known patatins isoforms have non-specific lipolytic activity, catalyzing the hydrolysis of phospholipids, glycolipids and mono and diacylglycerols [50,51]. With respect to patatins involved in plant pathogen interactions, Tonón et al. [52] have reported the purification of a patatin isoform that is induced after *P. infestans* infection in the apoplast of potato tubers and exerts antimicrobial activity toward *P. infestans* [52,53]. Additionally, Sharma et al. [54], have reported the purification, characterization and antifungal activity of a patatin isoform, named as patatin-J, from potato tubers. This protein was found to be induced in the stem tissue of potato after inoculation with *P. infestans*. These and our results suggest a positive correlation between patatin expression and the potato field resistance to *P. infestans*.

On the other hand, proteins from the SDS-PAGE bands that matched with annexin-like protein and enolase were only detected in the apoplast of infected tubers of the resistant potato cultivar. Animal annexins are involved in signal transduction, free cytosolic Ca^{2+} ($[Ca^{2+}]cyt$) homeostasis, exo- and endocytosis, membrane organization, cytoskeletal dynamics, cell cycle control, and water permeability [55—57]. Analogous functions in plants could place annexins at the center stage in signaling and adaptation. They are already implicated in cold, oxidative, saline, and abscisic acids (ABA) stress responses. Annexins can localize to the plant vacuolar membrane [58—60], the Golgi, and Golgi-derived vesicles [61]. Proteomic studies now show that plant and oomycete annexins exist in the cell wall as well as the cytoplasm [62,63]. In *Arabidopsis thaliana* several annexins have been associated with plant defense response. AnxAt1expression is up-regulated by salicylic acid, which implicates this annexin in pathogen defense responses [64]. Expression of *Arabidopsis* AnxAt4, tomato AnxLe34, and tobacco AnxNt12 also increases during pathogen attack but the functional significance remains unknown [65—67]. These antecedents agree with the results here obtained, and contribute to the idea that the increases in the expression of annexin-like proteins in potato tubers could be associated with the high field resistance of potato to *P. infestans*.

The essential and ubiquitous glycolytic enzyme enolase (EC 4.2.1.11) catalyzes the conversion of 2-phosphoglycerate to PEP. In higher plants, enolase, like other glycolytic enzymes, is present as multiple isoforms localized to the cytosol and to plastids [68,69]. In plants, several genes encoding proteins with functions in the basal metabolism, but not directly related to stress, were shown to be expressed at greater levels in response to drought or salinity stress [70,71]. Specifically, changes in gene expression of several enzymes involved in basic metabolic cellular pathways such as glycolysis and the Krebs cycle (e.g. enolase and triose phosphate isomerase) strongly suggest that these induced proteins play a role in the plant defense response [72—75]. Therefore, these antecedents and the results showed here suggest that the level of stress tolerance in the different plant genotypes could be associated with increases in the expression of pathogenesis and non pathogenesis related proteins.

Finally, two proteins (glutaredoxin and cyclophilin) related with the apoplast redox environment were detected only in infected tubers of the resistant cultivar. Previous reports show that among the many antioxidative mechanisms, oxidative stress can be buffered by the activation of chaperone-like proteins, such as cyclophilins (Cyps). Cyp expression in plant tissues increases response to different types of stress, such as heat shock and infection by pathogens [76]. Other reports show that plant cells contain numerous proteinaceous thiol components, including thioredoxin (Trx), glutaredoxin (Grx) and peroxiredoxin (Prx) proteins [77]. These thiol components have been implicated in a broad spectrum of cellular functions, ranging from moderating protein activity via the reversible oxidation of CysCys bridges, modulation of photosynthetic activity and to participation in stress defense [77]. The induction of cyclophillin and glutaredoxin showed in this work in the apoplast of tubers of cv. Innovator could be correlated with the higher field resistance to *P. infestans* of this potato cultivar.

3.3. Prediction of conserved secretion motifs in apoplastic hydrophobic secreted proteins

Another highlight of the obtained results is that approximately three quarters of the proteins putatively identified lacked the signal peptide known to be responsible for protein secretion. The possibility of contamination with intracellular components was minimized on the basis of the fact that the abundant intracellular Rubisco subunits were not detected in the IWFs by any of the highly sensitive spectrometric analysis performed and that less than 1% of total marker enzyme activity (α-mannosidase and glucose-6-P-dehydrogenase) was found in the IWFs of healthy or infected tubers. However, SecretomeP analyses revealed that 30% of proteins which were predicted to lack signal peptide are SecretomeP positive, suggesting unconventional protein secretion. Thus, our results support the hypothesis that plants might possess, as other eukaryotes do [33,78], an alternative route to the endoplasmic reticulum/Golgi dependent secretory pathway.

4. Material and methods

4.1. Plant and fungal material

Two potato cultivars with different degrees of field resistance against late blight disease were used. Whereas cv. Spunta is susceptible to *P. infestans*, cv. Innovator has a high level of field resistance ([16], http://www.argenpapa.com.ar). Potato tubers (*S. tuberosum* L. cv. Spunta and cv. Innovator) were provided by the Balcarce Experimental Station of the Instituto Nacional de Tecnologia Agropecuaria (INTA). *P. infestans* race R2 R3 R6 R7 R9, mating type A2, was obtained from the INTA Collection, Balcarce (Argentina). *P. infestans* was grown on potato tuber slices incubated in darkness at 18 °C. Seven days post-inoculation, mycelia was harvested in sterile water and stimulated to release zoospores by incubation for 2—3 h at 4 °C. After filtration through muslin, the resulting suspension was observed with a light microscope to quantify spores and sporangia. Concentration was adjusted to 10^5 sporangia ml^{-1}.

Tubers were washed and sterilized by immersion in 5% (w/v) sodium hypochlorite during 20 min. Sterile parenchyma disks (4–6 mm diameter, 10 mm thick) were prepared and inoculated with droplets containing 1.25×10^3 zoospores of *P. infestans* or sterile water and were incubated for 0 and 24 h at 18 °C in the dark in a moist chamber.

4.2. Preparation of total tuber extracts

Potato tuber disks were homogenized in 100 mM sodium acetate pH 5.2 containing 0.5% (w/v) sodium metabisulfite by applying 4 pulses of 10 s with 30 s intervals using a VirTis 45 homogenizer (The Virtis Co., Gardiner, New York, NY) set at speed 10. Homogenates were filtered through cheesecloth and centrifuged at 12,000 g for 20 min. The resulting supernatant represented the total tuber soluble extract (TTE).

4.3. Isolation of intercellular washing fluids (IWFs)

Intercellular washing fluids (IWFs) of potato tubers were obtained as previously described [24]. Tuber disks were washed four to five times with distilled water under gentle agitation. After washing, the tissue was immersed in a large excess of buffer containing 50 mM HCl–Tris (pH 8), 3.5% NaCl and 0.1% (v/v) β-mercaptoethanol and submitted to vacuum during three 10 s pulses separated by 30 s intervals. Tuber tissue was dried on filter paper, placed in a fritted glass filter inserted in a centrifuge tube and centrifuged for 20 min at $400 \times g$. The recovered IWF was used immediately or conserved at −20 °C.

As marker enzymes for the cytosolic and vacuolar fraction Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and α-Mannosidase (EC 3.2.1.24) activities were measured [25]. Glucose-6-phosphate dehydrogenase activity was measured for 15 min at 18 °C in 1.1 ml of a solution containing 50 μl extract, 65 mM $MgCl_2$, 3 mM glucose-6-phosphate, 1.2 mM $NADP^+$ and 85 mM triethanolamine (pH 7.6). The absorbance at 340 nm was continuously recorded. α-Mannosidase was measured using a standard assay [26]. The assay mixture contained in 0.5 ml, 50 μmol succinic acid adjusted to pH 5.0 with NaOH, and 0.3 μmol p-nitrophenol-∝-mannopyranoside as substrate. The reaction was incubated 5 min in buffer at 37 °C and the reaction started by the addition of substrate. It was stopped after 2 h by adding 0.8 ml of 1 M Na_2CO_3, after which the absorbance was measured at 405 nm.

4.4. Isolation of apoplastic hydrophobic proteins (AHP)

To obtain the Apoplastic Hydrophobic Proteins (AHP) present in the apoplast of healthy, wounded and infected tubers, IWFs from each treatment (corresponding to 3 g of fresh tissue) were dried in vacuum centrifuge (Savant AES 1010 Automatic Environmental Speed Vac®) and resuspended in 0.1% (v/v) TFA in water. Subsequently, samples were submitted to fast protein liquid chromatography (FPLC) in a PepRPC™ HR5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden) The column was equilibrated and washed with 0.1% (v/v) TFA in water, and then eluted with 0.1% TFA in 75% (v/v) acetonitrile for 15 min with a flow rate of 0.7 ml min^{-1}. Elution fractions obtained were pooled, concentrated in vacuum centrifuge (Savant AES 1010 Automatic Environmental Speed Vac®) and resuspended in 200 mM sodium acetate (pH 5.2) in presence of 75% (v/v) of acetonitrile.

4.5. Protein concentration

Protein concentration was measured by the bicinchoninic acid method [27], using bovine serum albumin (BSA) as standard. In the chromatographic profiles, the absorbance at 280 nm was recorded.

4.6. Protein analysis

IWFs and extracts containing AHPs from healthy, wounded and infected tubers were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% (p/v) acrylamide gel as described by Laemmli [28]. The samples were previously treated in denaturing buffer with β-mercaptoethanol before PAGE. Gels were stained with silver nitrate [29] or colloidal Coomasie Brilliant Blue G250 [30]. Densitometric analysis of SDS-PAGE bands was performed using Image J software (National Institutes of Health, Washington, USA, http://rsb.info.nih.gov/ij/).

All the bands showing a differential expression between cultivars and treatments (healthy, wounding and infection) were manually recovered, trypsin digested and the MALDI-TOF-MS spectra of the products were performed at the CEQUIBIEM (Universidad de Buenos Aires (UBA)-Universidad Nacional de La Plata (UNLP), Argentina. http://www.qb.fcen.uba.ar/cequibiem/). The following default parameters were set: an intensity or S/N threshold of 10; a local noise window width for each peptide of 250 m/z and a min peak width at full with half Max of 2.9 MS were analyzed in the 800–4000 range of mass to charge ratio (m/z). For calibrating each spectrum the following peaks of trypsin (m/z) were used: 842,51, 1045,56 and 2211,11. All the trypsin peptides were excluded as contaminants. Proteins were identified using the Mascot 1.9 search engine (Matrixscience, UK) on MSDB, NCBI and SwissProt database. A detailed analysis of peptide mass mapping data was performed using GPS Explorer™ software v 3.5 (Applied Biosystems), allowing the following parameters: species *Viridiplantae*, two missed cleavage, 2.5 Da precursor mass tolerance, as well as cysteine carbamidomethylation and methionine oxidation as possible modifications. The confidence in the peptide mass fingerprinting matches was based on the score level and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum. A threshold of 59 in Mascot score was statistically significant with $p < 0.05$. SignalP 3.0 software (www.cbs.dtu.dk/services/SignalP/) and SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP/) were used to predict motifs conserved in apoplastic proteins. SecretomeP is a sequence-based method that uses in the training of their neural networks six different features of secreted proteins. The mean of five resulting output values is the final score for each sequence and nonclassically secreted proteins must obtain a score exceeding the normal threshold of 0.5 to be considered SecretomeP positive [31]. This criterion was recently used for plant proteins [32,33].

4.7. Statistical analysis

Statistical analysis was performed by One-way ANOVA followed by Tukey test for comparisons among multiple groups. Analysis was done using SigmaStat 3.5 (http://www.systat.com/).

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