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Differential Expression of Potato Pathogenesis-related Proteins upon Infection with Late Blight Pathogen: A Case Study Expression of Potato Osmotin-like Protein

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ABSTRACT

Studies were performed to investigate the changes of potato pathogenesis-related proteins (PRPs) upon infection with late blight pathogen. Obtained data showed that in both resistant and susceptible potato cultivars, inoculated leaves with *Phytophthora infestans* showed a significantly higher amount of total protein than the healthy ones. In a bioassay experiment, the crude protein extracted from leaves of resistant potato cultivars showed the lowest, while those from susceptible ones indicated the highest fungal growth. SDS-PAGE analysis of acidic soluble proteins extracted from inoculated potato leaves at different periods of inoculation with *P. infestans* showed that nine proteins with molecular weight ranged from 12-45 kDa were increased gradually with time. RT-PCR analysis showed that in spite of the variation between tested potato cultivars in their resistance to late blight disease, the gene encoding osmotin-like protein (OSM-1 gene) existed at DNA level. At RNA level, an induction of OSM-1 gene expression was detected potato cultivars after inoculation with *P. infestans*. The expression of OSM-1 gene in the resistant cultivar occurred earlier (6 hpi) and stronger, while being induced later in the susceptible cultivar. Earlier and higher accumulation of PRPs in the resistant cultivars suggested that they were related to the defense mechanism against *P. infestans*. © 2010 Friends Science Publishers

Key Words: Potato; Pathogenesis-related proteins, Osmotin-like protein, Late bight disease

INTRODUCTION

Phytophthora infestans the causal agent of late blight disease, causes serious losses to potato and tomato crops worldwide and is probably the most important pathogen of both crops (Fry & Goodwin, 1997). This disease was the cause of the Irish potato famine in the 1840s, which resulted in about one million deaths and the emigration of about 1.5 million people to other parts of the world. Even now annual crop losses and fungicide costs amount to about US$4 billion throughout the world. While considerable efforts have been invested in plant breeding and genetic engineering, sources of resistance to late blight are still greatly needed (Solis *et al*., 2007).

Plants responding to infection by pathogens, or to various abiotic stresses, are induced to express a set of defense-related genes, such as the genes encoding pathogenesis-related proteins (PRPs). PRPs are a heterogeneous group of low molecular weight proteins selective solubility at low pH, resistant to proteolytic degradation and predominant accumulation in intercellular leaf spaces (Van-Loon, 1985; Bol *et al.,* 1990; Schroder *et al*., 1992). They accumulate rapidly at the intra-or extra-cellular level under various biotic and abiotic stimuli, including fungal, elicitor and physical or chemical treatments (Heller & Gessler, 1986; Van-Kan *et al*., 1992; Van-Loon & Van-Strien, 1999; Graham *et al*., 2003). Eleven PR-protein groups have been isolated and characterized in various plants (Kim *et al*., 2002). Among these proteins, PRP-2 is known for their β-1, 3-glucanase activity, PRP-3 for their chitinolytic activity, and PRP-5 for their osmo-permeabilization of fungal plasma membranes. The mode of action of members of PRP-1 family remains unclear (Wang *et al*., 2005).

The importance of PRPs to plant defense has been related to: (*a*) their rapid and early accumulation often associated with incompatibility, (*b*) their antimicrobial activity and (*c*) their ability to reduce symptoms development (Schroder *et al*., 1992; Wang *et al*., 2005). So constitutive expression of genes encoding PRPs is one of the strategies proposed to obtain a broad and durable level of resistance against different phytopathogenic fungi (Veronese *et al*., 1999). To check this possibility, we investigated the role of PR-proteins, in relation to resistance to late blight disease during *P. infestans*-potato interaction in resistant and susceptible cultivars.

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MATERIALS AND METHODS

Potato manipulation and plantation: Six potato cultivars (*S. tuberosum*) varied in their ability of resistance to late blight disease were used in this study (El_Komy, 2007); Hanna, Cara, (resistant) Spunta, (moderately resistant) Diamant, Lady-Rosetta and Lady-Olympia (susceptible). Potato cultivars were obtained from International Potato Center (CIP) Kafr El-zayat, Egypt. Potato plants were generated from high quality potato seed tubers and grown in clay pots containing mixtures of soil: sand: peat moth at a ratio of 4:4:1, respectively. Potato plants were kept in a growth chamber at 18ºC for 16 h photoperiod for 6 weeks prior to inoculation.

***Phytophthora infestans* isolate:** *Phytophthora infestans* were isolated from samples of potato plants showing symptoms of late blight. The pathogenic potentiality of the *P. infestans* was tested as described by Malcolmson (1979).

Determination of the changes in total protein content: Total soluble proteins extracted from inoculated (*in vivo*) potato leaves [resistant (Hanna, Cara) and susceptible (Lady-Rosetta & Diamant)] with *P. infestans* were prepared as described by Niderman *et al*. (1995). Studies were carried out 12, 24, 48, 56, 72 and 96 h post-inoculation (hpi). Un-inoculated potato leaves (treated with sterilized water & measured immediately) were served as control. Approximately, 5 g of potato leaves tissues of four potato cultivars (for each period) were immersed in liquid nitrogen, ground into a powder using a mortar and pestle and then suspended in 5 mL protein extraction buffer (0.1 M Phosphate buffer pH 7). The solution was centrifuged at 10,000×*g* for 20 min at 4°C and the supernatant was collected. The protein content was estimated according to Bradford (1976).

Antifungal activity of total protein extracted from potato leaves: Total soluble proteins extracted from inoculated (*in vivo*) potato leaves [resistant (Hanna, Cara) and susceptible (Lady-Rosetta & Diamant)] with *P. infestans* were prepared and adjusted to 250 µg mL^{-1} in phosphate buffer as described previously. Total protein was then sterilized by filtration through a 0.22 µm membrane filter (Millipore, Bedford, MA). The fungal growth bioassay was conducted according to the method of Ali and Reddy (2000). Sporangia were harvested from 3 weeks-old cultures of *P. infestans* by rinsing the plates with 10 mL sterilized distilled water. The sporangial suspension concentration was estimated using hoemocytometer to 1×10^5 sporangia mL^{-1}. Fifteen µL of sporangia suspension and 50 µL of liquid pea broth media were added to each well of a sterile 96-well titer plate. 250 µg of the extracted potato leaf proteins were applied in replicates of four wells for each treatment. Plates were covered with sterile lids and placed in a sealed polystyrene box with moistened filter paper to maintain humidity and incubated for 48 h at 18°C. Optical Density (OD) readings were taken at 630 nm over a 48 h time course by Universal automated microplate reader El_x 80.

Fungal growth rate = **[{**Final OD reading-initial OD reading (0 times)**}]**/Initial OD reading] X 100.

Determination of the changes in polypeptides profiles: Potato cultivars were inoculated with *P. infestans* as described previously. Acid soluble proteins were extracted as described by Tonon *et al*. (2002) as following, five grams of potato leaves cultivars were immersed in liquid nitrogen, ground into a powder using a mortar and pestle. The powder was re-suspended in 5 mL sodiumacetate buffer pH 5.2 (containing 0.1% β- mercaptoethanol & 0.1% w/v ascorbic acid) and centrifuged at 10000×*g* for 10 min. The protein content was estimated according to Bradford (1976). Acid soluble proteins extracted from leaves were analyzed using SDS-polyacrylamide gel electrophorisis (Laemmli, 1970).

Expression of Osmotin-Like Protein (PRP-5)

Molecular analysis at the structural level (DNA level): Total genomic DNAs were prepared from resistant (Hanna & Cara), moderately resistant (Nicola & Spunta) and susceptible (Lady-Olympia, Lady-Rosetta & Diamant) potato cultivars according to protocol of Griffith and Shaw (1998). Potato leaf tissue was processed by freezing with liquid nitrogen and was ground into a fine powder using a mortar and pestle. Approximately 100 mg of that powder was transferred to 1.5 mL micro-centrifuge tube and 600 µL of warm (65°C) modified CTAB extraction buffer (100 mM Tris-HCL [pH 8.0], 1.4 M NaCl, 2% CTAB [hexadecyltrimethylammonium bromide], 20 mM EDTA [sodium salt, pH 8.0]). Tubes were vortexes for 1-3 sec and incubated for 60-90 min, in water bath at 65°C. After that the sample was allowed to cool to room temperature for 5min. A volume of 700 µL chloroform/octanol (24:1) was added, the solution was gently mixed for 5-10 min. The mixture was centrifuged for 10 min at 8000×*g*. Six hundred micro-liters of upper, aqueous layer were transferred to clean1.5 mL micro-centrifuge tube and a volume of 600 µL of cooled isopropanol was added to precipitate the DNA. The mixture was centrifuged at 5000×*g* for 2 min at room temperature. The supernatant was decanted and 600 µL of 70% ethanol was added at room temperature and gently inverted the tube several times to wash the DNA. The mixture was centrifuged at 3000×*g* for 2 min at room temperature. Carefully the ethanol was aspirated using a pipette. The tube was inverted onto clean absorbent paper and air dried the pellet for 15 min. DNA pellet was re-suspended in 100 µL TE (10 mM Tris-HCL [pH 8.0], 1 mM EDTA [pH 8.0]) and stored at –20°C. DNA concentration was determined using spectrophotometer (Beckman DU-65) and was adjusted to 50 ng μL^{-1}.

PCR amplification was carried out in 25 µL volumes containing: 1 µL (50 pmol) of each primer, 0.3 µL *Taq* DNA polymerase (5 U μL^{-1}), 2.5 µL PCR buffer, 1 µL 10 mM Mg Cl_2, 1 µL 2.0 mM dNTPs (for each), 1 µL of template DNA (approximately 40 ng) and 17.2 µL sterilized distilled water. The PCR amplification conditions were initial denaturation at 94ºC for 5 min, followed by 35 cycles consisting of denaturation at 94ºC for 30 sec, annealing of

primer for 40 sec at 55°C, extension for 1.5 min at 72°C and finally addition of 3' terminal A for 10 min at 72°C. The specific primer for OSM-1 g gene was designed based (GenBank accession no AY770739), the primers sequence: *Forwared primer*: 5' ATG GGC TAC TTG ACA TCT TCTT 3'. *Reverse primer*: 5' TTA TGG GCA AAA AAC AAC CCT AT 3'. Amplified products were electrophoresed on 1.2% agrose gel containing 0.5 µL ethidium bromide with 1 X TBE running buffer. 1 kb DNA Ladder (New England Biolabs) with size marker, ranged from 500 to 10000 bp was used as a molecular size standard.

Molecular analysis at the functional level (RNA level): Leaves of resistant (Hanna) and susceptible (Lady-Rosetta) potato cultivars were inoculated (*in vivo*) with *P. infestans* as described previously. Studies were carried out 3, 6, 9, 12, 18, 24 and 30 hpi. Un-inoculated potato leaves (treated with sterilized water & measured immediately) were served as control. Total RNA was isolated from inoculated leaves using Biozol reagent (BioFlux). One hundred mg of the leaves were ground to a powder in a small mortar with liquid nitrogen and homogenized in 1 mL of Biozol solution. The lysate was transferred to a 1.5 mL tube and let stand for 5 min. at room temperature. Two hundred µL of chloroform was added and the mixture was centrifuged for 15 min at 12,000×*g*. The upper aqueous phase was transferred to a new tube and the volume was measured. While doing so, an equal volume of isopropanol was added, mixed and left for 10 min at room temperature. The precipitated RNA was collected by centrifugation for 10 min at 12,000×*g*. The pellet was air dried for 10-15 min and dissolved in sterilized distilled water (Ghazy, 2006).

DNA contamination was removed from RNA preparation using DNase I. The RNA, 10 to 50 µg, was transferred to a 1.5 mL tube. DNase I reaction buffer, 5.7 µL was added followed by the addition of 1 µL of DNase I (10 U). The solution was mixed well and incubated for 30 min at 37°C. Forty µL phenol: chloroform: isoamyl alcohol (25:24:1) was added, vortexed for 30 sec and the mixture was left for 10 min on ice. The mixture was centrifuged for 5 min. at 12,000×*g*. The upper aqueous phase was transferred to a new tube and 5 µL of 3 M sodium acetate was added to the aqueous phase followed by 200 µL of ethanol. Samples were incubated for 1hr at –20°C and centrifuged for 10 min. The supernatant was removed and the pellet was washed with 0.5 mL of 70% ethanol. The final pellet was dissolved in distilled sterilized water. RNA was electrophoresed to check the quality of the RNA (Ghazy, 2006).

Reverse transcription was done in 20 µL total volume in RT buffer 1 mM dNTPs, 0.5 µg Poly T Primer and 0.2 µg of total RNA. The following PCR program was used to synthesize the first strand cDNA: 70°C for 10 min. After the 5 min, 37°C for 5 min, 42°C for 60 min and 70°C for 10 min. After the 5min at 37°C the program was paused, 1 µL (200 U) of M-MuLV reverse transcriptase (fermentas) was added to each tube and the incubation was continued. At the end of the reverse transcription tubes were set on ice or stored at –20°C for later use (Ghazy, 2006). PCR amplification was performed as described previously, to amplify specific regions of the OSM-1 gene.

RESULTS

Changes in total protein content: The changes in protein content in leaves of resistant (Hanna & Cara) and susceptible (Lady-Rosetta & Diamant) potato cultivars after different periods of inoculation with *P. infestans* were determined. The protein content expressed as mg g^{-1} fresh weight. Data in Table I show that in both resistant and susceptible potato cultivars, inoculated leaves showed a higher amount of total protein than the healthy ones. Total protein content increased gradually with time. In period of 96 h post-inoculation the resistant potato cultivars showed a significant higher increase in the content of total proteins which reached 0.822 and 0.793 mg g^{-1} fresh weight in Hanna and Cara cultivars, respectively. The susceptible cultivars Diamant and Lady-Rosetta showed less increase in the content of total proteins, which reached 0.694 and 0.660 mg g^{-1} fresh weight, respectively.

Antifungal activity of total protein extracted from potato leaves: A bioassay experiment was adopted using microtiter dishes, in order to determine the effect of total protein extracted from leaves of resistant (Hanna & Cara) or susceptible (Lady-Rosetta & Diamant) potato cultivars, inoculated with *P. infestans* at different periods after inoculation on the growth of *P. infestans*. Sterilized protein solutions (250 µg) were added to a suspension containing sporangia of *P. infestans* (1×10^5 sporangia mL^{-1}) and pea broth media. Optical density (OD) readings were taken at 630 nm over a 48 h. Fungal growth rate was measured as the initial OD reading (0 times) subtracted from the final OD reading, divided by the initial OD reading X 100. As shown in Table II, there was significant difference between fungal growth on crude protein of un-inoculated resistant cvs. (Hanna & Cara) and the parallel susceptible potato cultivars (Diamant & Lady-Rosetta). Fungal growth rate on crude protein of un-inoculated Hanna, Cara, Lady-Rosetta and Diamant reached 18.0, 17.9, 22.5 and 21.9%, respectively. The percentage of the fungal growth was decreased in varied degrees with crude protein extracted from infected leaves with time in resistant or susceptible cultivars. Crude protein extracted from infected potato cultivars at 72 h post-inoculation, showed the lowest fungal growth rate (14.9%). Compared with control, the total protein extracted from inoculated leaves of potato cultivars caused the highest inhibition rates of fungal growth 41.7% in case of Hanna (72 hpi), 29.6% in case of Cara (48 hpi), 17.3% in case of Lady-Rosetta (96 hpi) and 24.4% in case of Diamant (72 hpi). The main effect of crude protein extracted from leaves of resistant potato cultivars Hanna and Cara showed the lowest percentages of fungal growth 14.1 and 14.4, respectively. Conversely crude protein extracted

Table I: Changes in protein content in leaves of resistant (Hanna & Cara) and susceptible (Lady-Rosetta & Diamant) potato cultivars inoculated with *P. infestans* expressed as mg g^{-1} fresh weight

Time post inoculation (hpi)	Potato cultivars								Mean effect of time
	Hanna		Cara		Lady Rosetta		Diamant		
	Mean*	**D (%)	Mean	D (%)	Mean	D (%)	Mean	D (%)	
***Control	0.56	-	0.55	-	0.56	-	0.56	-	0.556 f
12	0.66	+17.9	0.63	+14.5	0.59	+05.3	0.60	+07.1	0.621 e
24	0.81	+44.6	0.76	+38.1	0.69	+23.2	0.69	+23.2	0.731 d
48	0.92	+64.3	0.86	+56.4	0.70	+25.0	0.73	+30.4	0.801 c
72	1.02	+82.1	1.03	+87.3	0.77	+37.5	0.81	+44.6	0.909 a
96	0.97	+73.2	0.93	+69.0	0.68	+21.4	0.78	+39.3	0.836 b
Mean effect of cultivar	0.822 a		0.793 b		0.660 d		0.694 c		-

*Mean of 4 replicates
**D% = Difference % compared with healthy control among the same cultivar
***Control= un-inoculated leaves (treated with sterilized water and measured immediately)
-L.S.$D_{0.05}$ for interaction= 0.047
-Values followed by the same letter (s), are not significantly different at (P=0.05)

Table II: Antifungal activity of total protein extracted from resistant (Hanna & Cara) and susceptible (Lady-Rosetta & Diamant) potato leaves during 96 h post inoculation with *P. infestans* expressed as percentage of fungal growth rate

Time post inoculation (hpi)	Potato cultivars								Mean effect of time
	Hanna		Cara		Lady Rosetta		Diamant		
	Mean*	**D (%)	Mean	D (%)	Mean	D (%)	Mean	D (%)	
***Control	18.0 (25.1)	-	17.9 (25.0)	-	22.5 (28.3)	-	21.9 (27.8)	-	20.1 a (26.6)
12	16.7 (24.1)	- 07.2	15.6 (23.3)	- 12.9	21.9 (27.8)	- 02.7	20.3 (26.7)	- 07.9	18.0 b (25.0)
24	14.4 (22.3)	- 20.0	14.8 (22.5)	- 17.3	20.4 (26.8)	- 09.3	19.2 (25.9)	- 12.3	16.9 bc (24.2)
48	14.4 (22.2)	- 20.0	12.6 (20.8)	- 29.6	19.2 (25.9)	- 14.7	18.4 (25.3)	- 16.0	16.1cd (23.6)
72	10.5 (18.9)	- 41.7	12.8 (20.9)	- 28.5	18.8 (25.7)	- 16.4	17.6 (24.8)	- 24.4	14.9 d (22.6)
96	10.6 (19.0)	- 41.1	13.0 (21.1)	- 27.4	18.6 (25.5)	- 17.3	18.1 (25.2)	- 17.4	15.0 d (22.7)
Mean effect of cultivar	14.1 B (21.9)		14.4 B (22.3)		19.6 A (26.2)		19.3 A (25.9)		-

*Mean of 5 replicates
**D% = Difference % compared with control (0hpi) among the same cultivar
***Control= un-inoculated leaves (treated with sterilized water)
-Values between brackets are the arcsine square root of the transformation percentage of fungal growth rate
-L.S.$D_{0.05}$ for interaction= 2.30
-Values followed by the same letter (s), are not significantly different at (P=0.05)

from susceptible potato cultivars Lady-Rosetta and Diamant showed the highest percentages of fungal growth 19.6 and 19.3, respectively. It can be noticed that, crude protein extracted from leaves of resistant cultivars Hanna and Cara resulted in inhibitory effect on the growth of *P. infestans* than that from the susceptible potato cultivars.

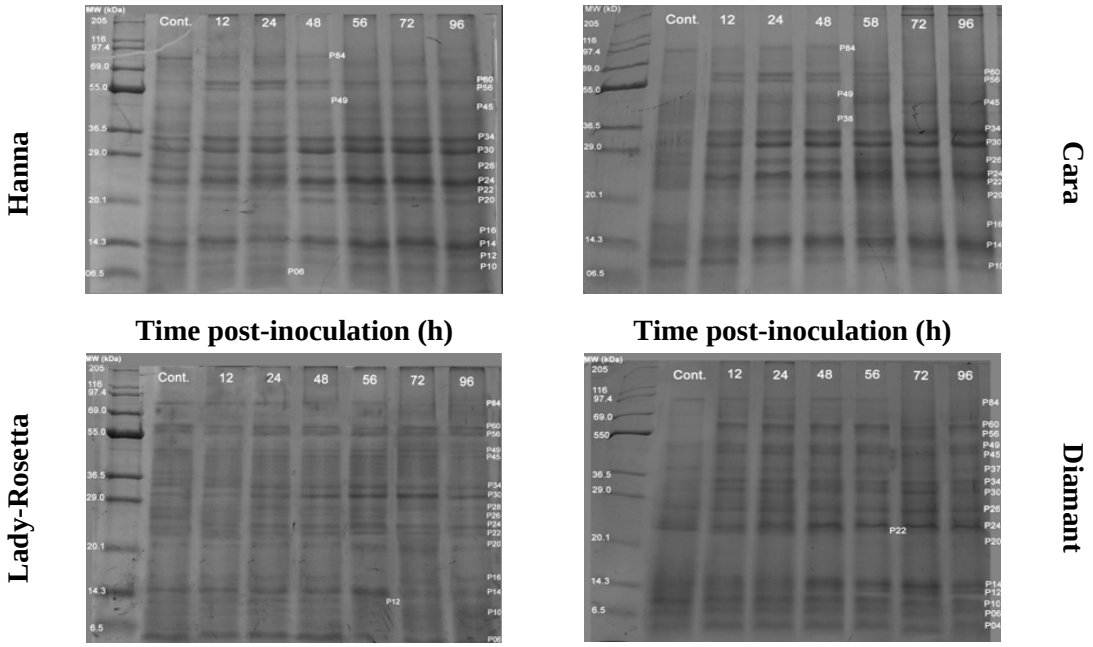
Changes in polypeptides profiles: Studies were carried out *in vivo* to investigate the changes in PR-proteins pattern in inoculate leaves of potato cultivars varied in their response to infection with *P. infestans* at different periods from inoculation. Studies were noticed at different periods 12, 24, 48, 56, 72 and 96 h post-inoculation. Un-inoculated treatment was served as a control. Acidic soluble proteins extracted from leaves of potato cultivars were electrophoresed under denaturing condition using sodium dodecylsulphate polyacrylamid gel electrophoresis (SDS-PAGE).

Analysis of acidic soluble proteins extracted from leaves of the resistant potato cultivars Hanna and Cara showed that, nine proteins with molecular weight ranged from 12-45 kDa were increased gradually with time. The molecular weight of these proteins were 12 (P12), 14 (P14), 20 (P20), 22 (P22), 24 (P24), 26 (P26), 30 (P30), 34 (P34) and 45 kDa (P45) (Fig. 1). The accumulation of these proteins started at 12 h post-inoculation and reached the maximum within 56–96 h post-inoculation. The accumulation of proteins with molecular weight of 56 (P56) and 60 kDa (P60) were increased after 12 h from inoculation and reached the highest increase at 24 h then slight decrease was detected. The high molecular weight protein (84 kDa) was decreased with time. Inoculated leaves of the susceptible potato cultivars (Lady-Rosetta & Diamant) showed a weak accumulation of P45, P34, P30, P26, P24, P22, P20 P14 and P12 (Fig. 1) The accumulation of these proteins started within 12 and 24 h post-inoculation.

These results indicated that, proteins with molecular weight ranging from 12-45 kDa were related to the defense mechanism against *P. infestans*. The quantity of the accumulating acid soluble protein depended on the resistance or susceptibility of potato cultivars. The accumulation of PR-proteins was earlier and higher in the resistant potato cultivars than in the susceptible ones.

Expression of osmotin-like protein (PRP-5): In this study, the regulation of OSM-1g gene, which encoding osmotin-

Fig. 1: Polyacrylamid gel electrophoresis (12%) of acid soluble protein showing accumulation of PR-proteins in leaves of the resistant (Hanna & Cara) and susceptible (Lady-Rosetta & Diamant) potato cultivars inoculated with *P. infestans* at different periods of inoculation. Loaded samples were adjusted to constant amount of protein (15 μg)



like protein (PRP-5) and induced during the *P. infestans* potato interaction in resistant and susceptible potato cultivars we investigated.

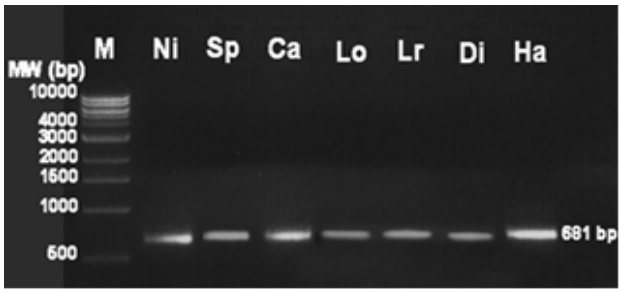
Molecular analysis at the structural level (DNA level): PCR was conducted to confirm the presence of the OSM-1g gene in the 7 potato cultivars varied in their resistance to late blight disease (Nicola, Spunta, Cara, Lady-Olympia, Lady-Rosetta, Diamant, Hanna), using specific primer, which is designed, based on the sequence encoding OSM-1g (GenBank accession no AY770739). As shown in (Fig. 2) the OSM-1g gene showed a band size of 681 bp in both resistant and susceptible cultivars such as Hanna (resistant) and Lady-Rosetta (susceptible). These results indicate that, in spite of the tested potato cultivars varied in their resistance to late blight disease, the gene encoding OSM-1g was existed at DNA level.

Molecular analysis at the functional level (RNA level): Expression of OSM-1g gene was tested on the RNA extracted from two potato cultivars differ in their levels of susceptibility to late blight disease, caused by *P. infestans* (isolate, P14); Hanna (resistant) and Lady-Rosetta (susceptible), before and after the inoculation (*in vivo*) with *P. infestans* across a different time course (post-inoculation). This was performed in order to study the time course and the spatial accumulation of this gene transcript in the potato-*P. infestans* interaction. RNA was extracted before inoculation and considered as control. After inoculation RNA extracts were obtained at 6, 9, 12, 18, 24 and 30 hpi.

As shown in Fig. 3 an induction of OSM-1g expression was detected in both cultivars (resistant &

Fig. 2: Polymerase chain reaction product for OSM-1g gene (681 bp) from different potato cultivars varied in their resistance to infection with *P. infestans*

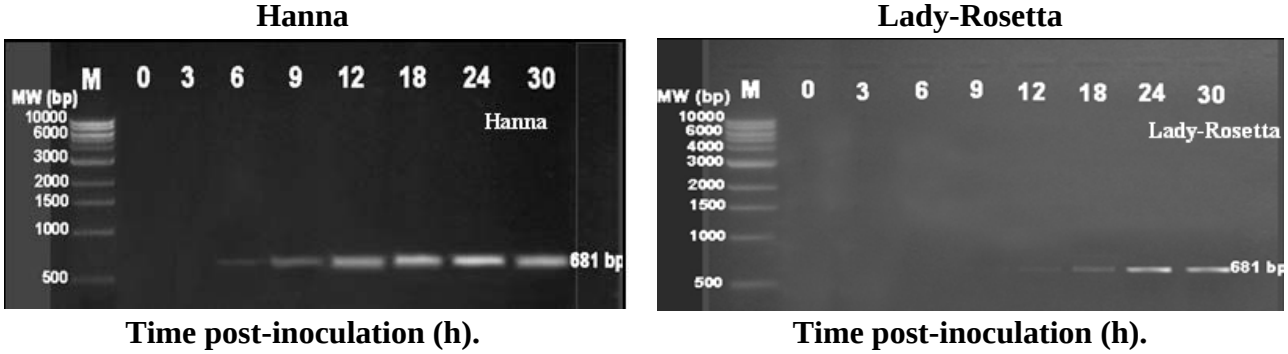
M = DNA Marker, Ni = Potato cv. Nicola (Moderately resistant), Sp = Potato cv. Spunta (Moderately resistant), Ca = Potato cv. Cara (Resistant), Lo = Potato cv. Lady Olympia (Susceptible), Lr = Potato cv. Lady Rosetta (Susceptible), Di = Potato cv. Diamant (Susceptible), Ha = Potato cv. Hanna (Resistant)



susceptible) after inoculation with *P. infestans*, as compared to the healthy controls. The expression of osmotin like protein was found to differ between resistant and susceptible potato cultivars. In case of resistant cultivar (Hanna), a detectable increase in OSM-1g transcription levels was observed at 6 hpi and was increase gradually at 9, 12, 18, 24 and 30 hpi. On the other hand in susceptible potato cultivar (Lady-Rosetta), gene transcripts was detected at 12 hpi and reached a maximum at 30 hpi. These results indicated that, OSM-1 g gene was related to the defense mechanisms against potato late blight. The expression of OSM-1 gene in the resistant cultivar occurred earlier and stronger, while being induced later in the susceptible cultivar.

Fig. 3: Reverse-transcriptase polymerase chain reaction analysis showing expression of the OSM-1g gene (681 bp) in leaves of the resistant (Hanna) and susceptible (Lady-Rosetta) potato cultivars inoculated with *P. infestans* at different periods of inoculation

- M = DNA marker, - 0 = Un-infected leaves (treated with sterilized water and measured immediately)



DISCUSSION

Two different types of late blight resistance are known, the qualitative and quantitative resistance. Qualitative resistance is mediated by R-genes, which leads to a race-specific hypersensitive response. This type of resistance was overcome quickly by new races of the pathogen having virulence alleles compatible with the resistance alleles in these cultivars. By contrast, non-race-specific resistance is controlled by many interacting genes that do not prevent infection, but slow down the development of the pathogen at individual infection sites on the plant. This type of resistance lasts longer, because it is race-non-specific and polygenic (Colon *et al*., 1995; Song *et al*., 2003; Ros *et al*., 2004), therefore current breeding strategies concentrate, on improving horizontal resistance.

Pathogenesis-related proteins (PRPs) accumulate rapidly at the intra-or extra-cellular level under various biotic and abiotic stimuli, including fungal, elicitor and physical or chemical treatments (Van-Loon, 1985; Bol *et al*., 1990; Schroder *et al*., 1992; Van-Kan *et al*., 1992; Van-Loon & Van-Strien, 1999; Graham *et al*., 2003). The proteomic studies carried through out this work indicated that, in both resistant and susceptible potato cultivars, leaves inoculated with *P. infestans* showed a significantly higher amount of total proteins than the healthy ones. In addition, total proteins content increased gradually with time. These results coincided, to a certain extent with the studies carried out by Hoegen *et al*. (2002) who showed that, inoculation of potato leaves with *P. infestans* led to massive accumulation of PRPs. The total amount of proteins increased due to infection from about 0.1 to 1 mg mL^{-1}. Also we found that, in period of 96 h post-inoculation the resistant potato cultivars showed a significant higher increase in the content of total proteins, which reached 0.822 and 0.793 mg g^{-1} fresh weight in Hanna and Cara cultivars, respectively. On the other hand, the susceptible cultivars showed less increase in the content of total proteins. Such results are in harmony with those reported by Schroder *et al*. (1992) and Tonon *et al*. (1998 & 2002), who indicated that the absolute amount of accumulated PRPs was higher in incompatible interaction than the compatible ones.

PRPs s have been shown to have antifungal activity *in vitro*, among them PRP-2, which are known for their β-1, 3-glucanase activity PRP-3, which are known for their chitinolytic activity and PRP-5, which are known for their osmo-permeabilization of fungal plasma membranes, while the mode of action of members of the PRP-1 family remains unclear (Schroder *et al*., 1992; Wang *et al*., 2005). In the present work, a bioassay experiment was adopted using microtiter dishes, in order to determine the effect of total protein extracted from leaves of resistant (Cara & Hanna) or susceptible (Lady-Rosetta & Diamont) potato cultivars, inoculated with *P. infestans* at different periods after inoculation on the growth of *P. infestans*. Results showed that, the percentage of the fungal growth was decreased in varied degrees with crude protein extracted from infected leaves with time in resistant or susceptible cultivars. The main effect of crude protein extracted from leaves of resistant potato cultivars showed the lowest percentage of fungal growth. Conversely crude protein extracted from susceptible potato cultivars showed the highest percentage of fungal growth. Based on these results, it can be noticed that, crude protein extracted from leaves of resistant cultivars resulted in inhibitory effect on the growth of *P. infestans* than that from the susceptible potato cultivars. Niderman *et al.* (1995) tested three distinct basic 14 kDa proteins, P14a, P14b and P14c, isolated from tomato leaves infected with *P. infestans*. They exhibited antifungal activity against *P. infestans* both *in vitro* (inhibition of zoospore germination) and *in vivo* with a tomato leaf disc assay. Osmotin has been shown to have antifungal activity *in vitro* (Liu *et al*., 1994; Ibeas *et al*., 2000 & 2001; Narasimhan *et al*., 2001) and when tested in combination with chitinase an β-1, 3-glucanase, showed enhanced lytic activity (Vigers, 1992; Sela-Buurlage *et al*., 1993; Lorito *et al*., 1996; Jeun & Buchenaue, 2000; Kyu Hong *et al*., 2004). AP24 is one of the PR-proteins, which also accumulated in tomato plants infected by *P. infestans*. AP24 inhibits hyphal growth and cause lysis of sporangia of *P. infestans* at conc. >40 nM and

severely inhibited hyphal growth at conc. >400 nM (Woloshuk *et al*., 1991).

During plant-pathogen interaction accumulation of PRPs was detected as a response to infection. These PRPs included; four chitinase (26, 27, 30, 32 kDa) (Lawrence *et al*., 1996), tow β-1, 3- glucanase (33 & 35 kDa) (Lawrence *et al*., 1996; Anfoka & Buchenauer, 1997; Tonon *et al*., 2002), tow osmotin isoform (22 & 24 kDa) (Woloshuk *et al*., 1991; Takemoto *et al*., 1997), PRP-1 type protein (14 kDa) (Niderman *et al*., 1995; Jeun, 2000; Hoegen *et al*., 2002; JeumKyu & ByungKook, 2002), a 45 kDa protein (Fischer *et al*., 1989) and a 69 kDa protein, which may be similar or identical to the alkaline endoprotinase (Lawrence *et al*., 1996). During *in vivo* studies we investigate the changes in protein pattern in infected leaves of potato cultivars, which varied in their response to the infection with *P. infestans* at different periods from inoculation. Also we examined the relationship between the accumulation of PRPs and potato's late blight resistance. SDS-PAGE analysis of acidic soluble proteins extracted from leaves of the resistant potato cultivars Hanna and Cara showed that, nine proteins with molecular weight ranged from 12-45 kDa were increased gradually with time. The molecular weight of these proteins were 12 (P12), 14 (P14), 20 (P20), 22 (P22), 24 (P24), 26 (P26), 30 (P30), 34 (P34) and 45 kDa (P45). The accumulation of these proteins started at 12 hpi and reached the maximum within 56-96 hpi. Also, the accumulation of proteins with molecular weight of 56 (P56) and 60 kDa (P60) were increased after 12 h from inoculation and reached the highest increase at 24 h then slight decrease was detected. On the other hand, inoculated leaves of the susceptible potato cultivars showed a weak accumulation of P45, P34, P30, P26, P24, P22, P20 P14 and P12 proteins. The accumulation of these proteins started within 12-24 h post-inoculation. These results indicated that, proteins with molecular weight ranging from 12-45 kDa were related to the defense mechanism against *P. infestans*. The quantity of the accumulating acid soluble proteins depended on the resistance or susceptibility of potato cultivars. The accumulation of PRPs was earlier and higher in the resistant potato cultivars than in the susceptible ones. Such results are in harmony with those reported by Schroder *et al*. (1992), Tonon *et al*. (1998 & 2002), JeumKyu and ByungKook (2002) and Kyu Hong *et al*. (2004) who showed that, strong accumulation of PRPs occurred in response to infection by *P. infestans*. PRPs induced at lower levels in the susceptible cultivars than those observed in resistant cultivars.

Constitutive expression of genes encoding PRPs are one of the strategies proposed to obtain a broad and durable level of resistance to different phytopathogenic fungi (Veronese *et al*., 1999). In the present study we investigated the regulation of OSM-1g gene, which encoding osmotin-like protein (PRP-5) during th *P. infestans*-potato interaction in resistant and susceptible potato cultivars. Results showed that, in spite of the tested potato cultivars varied in their resistance to late blight disease, the gene encoding OSM-1g was existed at DNA level. At RNA level results showed that, OSM-1g gene was related to the defense mechanisms against potato late blight disease. The expression of OSM-1 gene in the resistant cultivar occurred earlier (6 hpi) and stronger, while being inducted later (12 hpi) in the susceptible cultivar. Such results are in agreement with those reported by Kyu Hong *et al*. (2004) Wang *et al*. (2005), who demonstrated that expression of osmotin gene was higher and earlier in the incompatible interaction than the compatible interaction. Differences in defense response production between these potato cultivars might be due to differences in the expression of R gene, which are considered to play a role in specific pathogen recognition (Trognitz *et al*., 2002; Polkowaska-Kowalczyk *et al*., 2004). Trognitz *et al*. (2002) found differences in the number of osmotin gene copies in the genome among potato cultivars. Resistant cultivar had high copy numbers compared with susceptible ones. In a summary earlier and higher accumulation of PRPs in the resistant cultivars suggested that they were related to the defense mechanism against *P. infestans*.

REFERENCES

Ali, G.S. and A.S. Reddy, 2000. Inhibition of fungal and bacterial plant pathogen by synthetic peptides: *in vitro* growth inhibition, interaction between peptides and inhibition of disease progression. *Mol. Plant-Microbe. Interact.*, 13: 847–859

Anfoka, G. and H. Buchenauer, 1997. Systemic acquired resistance in tomato against *Phytophthora infestans* by pre-inoculation with tobacco necrosis virus. *Physiol. Mol. Plant Pathol*., 50: 85–101

Bol, J.F., H.J.M. Linthorst and B.J.C. Cornelissen, 1990. Plant pathogenesis-related proteins induced by virus infection. *Annl. Rev. Phytopathol*., 28: 113-138

Bradford, M.M., 1976. A rapid and sensitive methods for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* 72: 248–254

Colon, L.T., L.J. Turkensteen, W. Prummel, D.J. Budding and J. Hoogendoorn, 1995. Durable resistance to late blight (*Phytophthora infestans*) in old potato cultivars. *European J. Plant Pathol*., 101: 387–397

El_Komy, M.H., 2007. The role of certain resistance factors affecting the development of late blight disease of potato. *Ph.D. Thesis*, p: 220. Faculty of Agriculture, Alexandria University, Egypt

Fry, W.E. and S.B. Goodwin, 1997. Resurgence of the Irish potato famine fungus. *Biol. Sci.*, 47: 363–371

Fischer, W., U. Christ, M. Baumgartner, K.H. Erismann and E. Mosinger, 1989. Pathogenesis-related proteins of tomato: II. Biochemical and immunological characterization. *Physiol. Mol. Plant Pathol*., 35: 67–83

Ghazy, A.I.A., 2006. Genetic studies using biotechnology for the improvement of potato. *Ph.D. Thesis*, p: 123. Faculty of Agriculture Alexandria University, Egypt

Graham, M.Y., J. Weidner, K. Wheeler, M.L. Pelow and T.L. Graham, 2003. Induced expression of pathogenesis-related protein genes in soybean by wounding and the *Phytophthora sojae* cell wall glucan elicitor. *Physiol. Mol. Plant Pathol*., 63: 141–149

Griffith, G.W. and D.S. Shaw, 1998. Polymorphisms in *Phytophthora infestans* four mitocondrial haplotypes are detected after PCR amplification of DNA from pure culture or from host lesions. *Appl. Environ. Microbiol*., 64: 4007–4014

Heller, W.E. and C. Gessler, 1986. Induced systemic resistance in tomato plants against *Phytophthora infestans*. *J. Phytopathol*., 116: 323–328

Hoegen, E., A. Stromberg, U. Pihlgren and E. Kombrink, 2002. Primary structure and tissue specific expression of the pathogenesis related protein PR-1b in potato. *Mol. Plant Pathol*., 3: 329–345

Ibeas, J.I., H. Lee, B. Damsz, D.T. Prasad, J.M. Pardo and P.M. Hasegawa, 2000. Fungal cell wall phosphomannans facilitate the toxic activity of a plant PR-5 protein. *Plant J*., 23: 375–383

Ibeas, J.I., D. Yun, B. Damsz, M. Narasimhan, Y. Uesono, C. Ribas, H. Lee, P.M. Hasegawa, R.A. Bressan and J.M. Pardo, 2001. Resistance to the plant PR-5 protein osmotin in the model fungus *Saccharomyces cerevisiae* is mediated by the regulatory effects of SSD1 on cell wall composition. *Plant J*., 25: 271–281

JeumKyu, H. and H. ByungKook, 2002. Temporal and subcellular localization of PR-1 proteins in tomato stem tissues infected by virulent and avirulent isolates of *Phytophthora capsici. Protoplasma*, 219: 131–139

Jeun, Y.C., 2000. Immunolocalization of PR-protein P14 in leaves of tomato plants exhibiting systemic acquired resistance against *Phytophthora infestans* induced by pretreatment with 3-aminobutryic acid and preinoculation with tobacco necrosis virus. *Zeitschrift-fur-Pflanzenkrankheiten-und-Pflanzenschutz*, 107: 352–367

Jeun, Y.C. and H. Buchenauer, 2000. Infection structures and localization of the pathogenesis-related protein AP24 in leaves of tomato plants exhibiting systemic acquired resistance against *P. infestans* after pre-treatment with 3-aminobutyric acid or tobacco necrosis virus. *J. Phytopathol*., 149: 141–153

Kim, H., J.H. Mun, B. Byun, H. Hwang, Y. Kwon and S.G. Kim, 2002. Molecular cloning and characterization of the gene encoding osmotin protein in Petunia hybrida. *Plant Sci.*, 162: 745–752

Kyu Hong, J., H. Won Jung, B.K. Lee, S.C. Lee, Y.K. Lee and B.K. Hwang, 2004. An osmotin-like protein gene, CAOSM1, from pepper: differential expression and in situ localization of its mRNA during pathogen infection and abiotic stress. *Physiol. Mol. Pathol.*, 64: 301–310

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680–785

Lawrence, C.B., H.A.J. Joosten and S. Tuzun, 1996. Differential induction of pathogenesis related proteins in tomato by *Alternaria solani* and the association of a basic chitinase isozyme with resistance. *Physiol. Mol. Plant Pathol*., 48: 361–377

Liu, D., K. Raghothama, G.P. Hasegawa and A. Bressan, 1994. Osmotin overexpression in potato delays development of disease symptoms. *Proc. Natl. Acad. Sci. USA*, 91: 1888–1892

Lorito, M., S.L. Woo, M. D'Ambrosio, G.E. Harman, C.K. Hayes, C.P. Kubicek and F. Scala, 1996. Synergistic interaction between cell wall degrading enzymes and membrane affecting compounds. *Mol. Plant-Microbe Interact*., 9: 206–213

Malcolmson, J.F., 1979. Isolation and identification of races of *Phytophthora infestans* from breeder's assessment plots. *Trans. British Mycol. Soc.*, 73: 155–156

Narasimhan, M.L., B. Damsz, M.A. Coca, J.I. Ibeas, D.J. Yun and J.M. Pardo, 2001. A plant defense response effector induces microbial apoptosis. *Mol. Cell*., 8: 921–930

Niderman, T., I. Genetet, T. Bruyere, R. Gees, A. Stintzi, M. Legrand, B. Fritig and E. Mosinger, 1995. Pathogenesis-related PR-1 proteins are antifungal. Isolation and characterization of three 14 kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. *Plant Physiol*., 108: 17–27

Polkowaska-Kowalczyk, L., B. Wielgat and U. Maciejewska, 2004. The elicitor-induced oxidative processes in leaves of *Solanum* species with differential polygenic resistance to *Phytophthora infestans*. *J. Plant Physiol*., 161: 913–920

Ros, B., F.I.T.Z. Thummler and G. Wenzwl, 2004. Analysis of differentially expressed genes in a susceptible and moderately resistant potato cultivar upon *Phytophthora infestans* infection. *Mol. Plant Pathol*., 5: 191–201

Schroder, M., K. Hahlbrock and E. Kombrink, 1992. Temporal and spatial patterns of β-1, 3-glucanase and chitinase induction in potato leaves infected by *Phytophthora infestans*. *Plant J*., 2: 161–172

Sela-Buurlage, M.B., A.S. Ponstein, S.A. Bres-Vloemans, L.S. Melchers, P.J.M. Van Den Elzen and B.J.C. Cornelissen, 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1, 3-glucanases exhibit antifungal activity. *Plant Physiol*., 101: 857–863

Solis, J., G. Medrano and M. Ghislain, 2007. Inhibitory effect of a defensin gene from the Andean crop maca (*Lepidium meyenii*) against *Phytophthora infestans*. *J. Plant Physiol*., 164: 1071–1082

Song, J., J.M. Bradeen, S.K. Naess, J.A. Raasch, S.M. Wielgus, G.T. Haberlach, J. Liu, H. Kuang, S. Austin-Phillips, C.R. Buell, J.P. Helgeson and J. Jiang, 2003. Gene RB cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. *PNAS*, 100: 9128–9133

Takemoto, D., K. Furuse, N. Doke and K. Kawakita, 1997. Identification of chitinase and osmotin-like protein as actin-binding proteins in suspension-cultured potato cells. *Plant Cell Physiol.*, 38: 441–448

Tonon, C., A. Andreu, M. Aued, M. Van Damme, M. Huarte and G. Daleo, 1998. Defense reaction in two potato cultivars following infection with two races of *Phytophthora infestans*. *Potato Res*., 41: 319–325

Tonon, C., G. Guevara, C. Oliva and G. Daleo, 2002. Isolation of a potato acidic 39 kDa β-1, 3-glucanase with antifungal activity against *Phytophthora infestans* and analysis of it's expression in potato cultivars differing in their degrees of field resistance. *J. Phytopathol*., 150: 189–195

Trognitz, F., P. Manosalva, R. Gysin, D. Nino-Liu, R. Simon, M. Herrera, B. Trognitz, M. Ghislain and R. Nelson, 2002. Plant defense genes associated with quantitative resistance to potato late blight in *Solanum phureja* x *Dihaploid S. tuberosum* hybrids. *Mol. Plant-Microbe Interact*., 15: 587–597

Van-Kan, J.A.L., M.H.A.J. Joosten, C.A.M. Wagemakers, G.C.M. Van Den Berg Velthuis and P.J.G.M. De Wit, 1992. Deferential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato by virulent and avirulent races of *Cladosporium fulvum*. *Plant Mol. Biol*., 20: 513–527

Van-Loon, L.C., 1985. Pathogenesis-related proteins. *Plant Mol. Biol.*, 4: 111–116

Van-Loon, L.C. and E.A. Van-Strien, 1999. The families of pathogenesis related proteins, their activities and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol*., 55: 85–97

Veronese, P., P. Crino, M. Tucci, F. Colucci, D.J. Yun, M.P. Hasegawa, R.A. Bressan and F. Saccardo, 1999. Pathogenesis-related proteins for the control of fungal diseases of tomato. *Genetics and Breeding for Crop Quality and Resistance Proceedings of the XV-EUCARPIA Congress*, pp: 15–24. Viterbo, Italy, September, 20-25, 1999

Vigers, A.J., S. Wiedemann, W.K. Roberts, M. Legrand, C.P. Selitrenniko and B. Fritig, 1992. Thaumatin-like pathogenesis-related proteins are antifungal. *Plant Sci*., 83: 155–161

Wang, X., A. El Hadrami, L.R. Adam and F. Daayf, 2005. Local and distal gene expression of *pr-1* and *pr-5* in potato leaves inoculated with isolates from the old (US-1) and the new (US-8) genotypes of *Phytophthora infestans* (Mont.) de Bary. *Environ. Exp. Bot*., 1–11

Woloshuk, C.P., J.S. Meulenho, M. Sela-Buurlage, P.J.M. Van Den Elzen and B.J.C. Cornelissen, 1991. Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. *Plant Cell*, 3: 619–628

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