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A proteomic approach to studying plant response to crenate broomrape (*Orobanche crenata*) in pea (*Pisum sativum*)

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Abstract

Crenate broomrape (*Orobanche crenata*) is a parasitic plant that threatens legume production in Mediterranean areas. Pea (*Pisum sativum*) is severely affected, and only moderate levels of genetic resistance have so far been identified. In the present work we selected the most resistant accession available (Ps 624) and compared it with a susceptible (Messire) cultivar. Experiments were performed by using pot and Petri dish bioassays, showing little differences in the percentage of broomrape seed germination induced by both genotypes, but a significant hamper in the number of successfully installed tubercles and their developmental stage in the Ps 624 compared to Messire.

The protein profile of healthy and infected *P. sativum* root tissue were analysed by two-dimensional electrophoresis. Approximately 500 individual protein spots could be detected on silver stained gels. At least 22 different protein spots differentiated control, non-infected, Messire and Ps 624 accessions. Some of them were identified by MALDI-TOF mass spectrometry and database searching as cysteine proteinase, β-1,3-glucanase, endochitinase, profucosidase, and ABA-responsive protein. Both qualitative and quantitative differences have been found among infected and non-infected root extracts. Thus, in the infected susceptible Messire genotype 34 spots were decreased, one increased and three newly detected, while in Ps 624, 15 spots were increased, three decreased and one newly detected. In response to the inoculation, proteins that correspond to enzymes of the carbohydrate metabolism (fructokinase, fructose-bisphosphate aldolase), nitrogen metabolism (ferredoxin-NADP reductase) and mitochondrial electronic chain transport (alternative oxidase 2) decreased in the susceptible check, while proteins that correspond to enzymes of the nitrogen assimilation pathway (glutamine synthetase) or typical pathogen defence, PR proteins, including β-1,3-glucanase and peroxidases, increased in Ps 624. Results are discussed in terms of changes in the carbohydrate and nitrogen metabolism an induction of defence proteins in response to broomrape parasitism.

Keywords: Parasitic plants; *Orobanche crenata*; Broomrape; *Pisum sativum*; Pea; Root proteomics; Pea proteomics; Two-dimensional gel electrophoresis; Peptide mass fingerprinting; MALDI-TOF

1. Introduction

Approximately 1% of flowering plants (nearly 4000 species) are parasitic on other plant species, with *Striga*, *Orobanche* and *Cuscuta* spp. being the most damaging, as they infect crops, causing significant yield losses (Riches and Parker, 1995). Several broomrapes (*Orobanche* spp.) are serious constraints for important crops, such as *O. cumana* on sunflower, *O. ramosa* and *O. aegyptiaca* on tomato and potato, and *O. crenata* on legumes. *O. crenata* is the most dangerous and widespread broomrape in the Mediterranean region and western Asia, being a serious limitation for the cultivation of a number of legume crops, including faba bean, pea, lentil, and various forage legumes (Rubiales, 2001, 2003). Several control

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strategies have been proposed and employed, but none has provided complete protection (Rubiales et al., 2003a). Breeding for resistance is thought to be the most desirable control strategy, although in some cases, like pea, little resistance has so far been identified. Resistance to *O. crenata* in legumes is a quantitative, multigenic character, making breeding resistance a difficult task (Rubiales et al., 2003a,b). The level of resistance can be increased by a combination of several mechanisms of defence in the same cultivar, which could also increase the durability of resistance, as monogenic resistance has proven to be rapidly overcome by the parasite (Rubiales, 2003). A range of host plant strategies for parasite resistance could be proposed, each one directed at interrupting specific developmental stages of the parasite biological cycle: germination, haustorial induction, attachment, penetration and connection to the vascular system, development, emergence, and flowering (Fig. 1; Jorrín et al., 1999; Rubiales et al., 2004).

Studies on parasitic angiosperms and their interaction with host plants are limited compared with the intensive research carried out into other symbiotic associations involving plants. At the molecular level, research has been mainly focused on identifying and characterizing the host signals that induce germination and haustorium induction (Yoder, 1999; Galindo et al., 1992). A number of reports reveal that typical plant defence responses against pathogenic microorganisms are also induced in response to parasitic plant infection. These include phytoalexin induction (Serghini et al., 2001), peroxidase activity increase, lignification and cell-wall phenolic deposition (Goldwasser et al., 1999), pathogenesis-related protein (Joel and Portnoy, 1998), and 3-hydroxy-3-methylglutaryl CoA reductase encoding gene induction (Westwood et al., 1998).

The "*omic*" experimental approaches will be of great value in obtaining a more global, comprehensive knowledge of the molecular events mediating plant–parasitic plant interactions and those supporting host plant response and resistance. Gene expression changes have been investigated in the roots of *Arabidopsis thaliana* seedlings infected by *O. ramosa* by using a subtractive hybridization technique (Werner et al., 2001; Vieira dos Santos et al., 2003). Of recent application to plants, compared with other organisms, proteomics is starting to provide information and a deeper understanding of different aspects of the plant biology (Canovas et al., 2004).

Studies describing the mitochondrial, chloroplast, root and symbiosome pea proteome have recently been published (Peltier et al., 2000; Bardel et al., 2002; Saalbach et al., 2002; Repetto et al., 2003). The only paper so far published on the application of proteomics on parasitic plant research is focused on patterns of protein synthesis during haustorial development of *Striga hermonthica* (Stranger et al., 1995).

As a global approach to better understand the mechanisms involved in pea resistance to *O. crenata*, we aimed at comparing the proteins expressed in response to infection in genotypes differing in their resistance against *Orobanche* plant parasite. For this purpose, Messire and Ps 624 pea genotypes were first investigated for *O. crenata* tolerance or susceptibility. Root proteins differentially expressed between the two infected and non-infected pea genotypes were further analysed by using a proteomic approach including two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry.

2. Results

2.1. Differences in resistance to broomrape between Messire and Ps 624 genotypes

Differences in resistance to broomrape between Messire and Ps 624 genotypes were evaluated by pot and Petri dish experiments (Fig. 1). There were little differences in the percentage of broomrape seed germination among Messire and Ps 624, but the number of established tubercles and emerged broomrapes was much lower in Ps 624 than in Messire (Table 1).

2.2. Two-dimensional gel electrophoresis and MALDI-TOF protein analysis

2.2.1. Protein differences among the two pea genotypes

The protein profiles of healthy *P. sativum* root tissues were analysed by two-dimensional electrophoresis with 17 cm, 3–10 pH linear gradient IEF strips, as first dimension, and 12% polyacrylamide home-made SDS–PAGE, as the second one. As determined following the use of the PD-Quest^{TM} software, over 500 individual protein spots could be detected on silver-stained gels (Figs. 2–4). Within each pea genotype, based on spot number and volumes, the protein patterns were reproducible among replicates from different tissue extraction samples, with the majority of spots being displayed within the 4–7 pH and 20–60 kDa molecular weight ranges. Nevertheless, qualitative as well as quantitative differences were found between the non-infected Messire and Ps 624. After PD-Quest analysis only spot differences (presence/absence as well as quantitative) consistently manifested in all the replicates and visually confirmed were considered and included in Table 2. Changes included differences in spot intensity as well as the occurrence of apparently new ones. Twenty-two protein spots were differentially displayed between the two non-infected genotypes (Fig. 2 and Table 2). This is in agreement with the ability of the 2-DE technique to successfully detect genetic diversity within herbaceous and woody plant species (Thiellement et al., 1999).

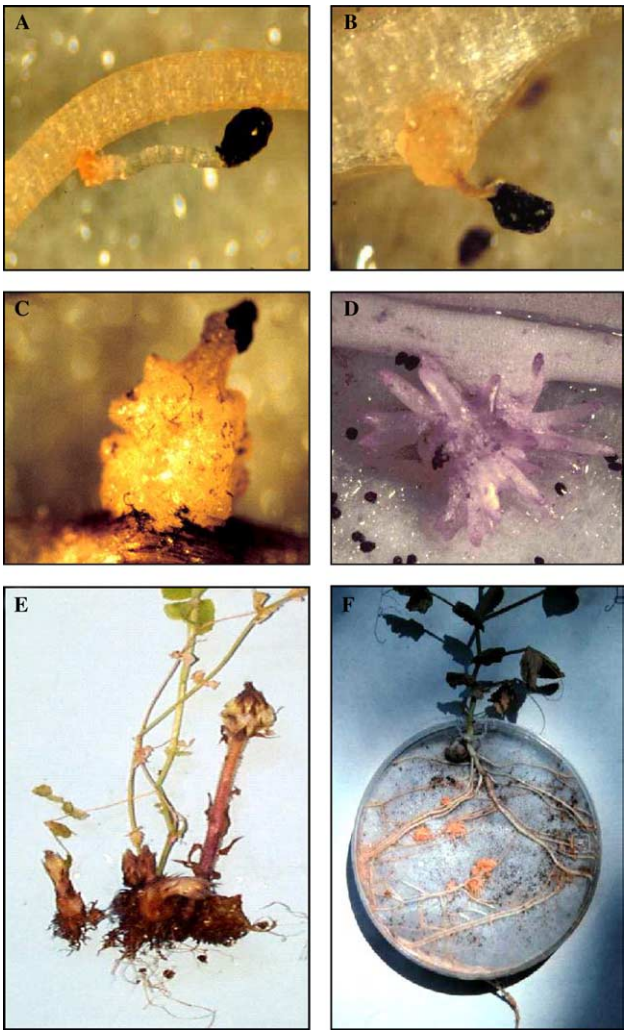

Fig. 1. Developmental stages of the *Orobanche* biological cycle, including germination (A), attachment (B) tubercle development (C–D) and shoot emergence (E). Pictures correspond to the pea (Messire)–*O. crenata* interaction and were taken from a dish bioassay (F).

Table 1
O. crenata seed germination, tubercle formation and emergence on roots of pea (*P. sativum*), as determined by pot and Petri dish experiments

Accession	Number of installed broomrapes per host plant[a]	% Broomrapes seed germination[b]	Number of broomrapes tubercles per host plant[c]
Messire	26	50	250
Ps 624	4*	65[d]	90[d]

[a] As determined by pot experiments. Included the 1–7 broomrape developmental stages (Ter Borg et al., 1994). Values are mean of 10 independent replicates.

[b] As determined by using the Petri dish bioassay. Germination at 0–3 mm from host root was estimated and expressed as percentage of the total seeds. (Rubiales et al., 2004). Values are mean of four independent replicates.

[c] As determined by using the Petri dish bioassay. Included the 1–4 developmental stages (Ter Borg et al., 1994). Values are mean of four independent replicates.

[*] Means significantly different ($P < 0.05$, Duncan test).

All these spots were sampled from micro-preparative gels to be analysed by MALDI-TOF mass spectrometry. Only 5 out of the 22 proteins could be assigned with a function (Table 3). Spots 1, 6, 12, 13 and 15 matched with a cysteine proteinase, a glucan endo-1,3-glucosidase precursor, an endochitinase A2 precursor, a profucosidase and an ABA-responsive protein, respectively. The glucan β-1,3-glucosidase precursor (spot 6) and the ABA-responsive protein (spot 15) were only detected in the Ps 624 root extracts. The endochitinase A2 precursor and the cysteine proteinase were detected in a greater amount in the Ps 624 genotype while the profucosidase was more abundant in Messire pea roots.

2.2.2. Protein changes in the susceptible Messire genotype in response to O. crenata inoculation

Several protein modifications were detected in the root extracts of the susceptible Messire genotype in response to *O. crenata* inoculation including a decrease (34 spots) or an increase (one spot) in abundance. Three spots seemed to have been newly induced (Fig. 3 and Table 4). Following gel excision of the 38 spots of interest and MALDI-TOF analysis, seven proteins were identified (Table 5). They corresponded to enzymes of the carbohydrate metabolism, nitrogen assimilation and of the mitochondrial electronic transport pathway. They included a fructokinase (spot 33), a fructose-bisphosphate aldolase (spots 41 and 42), a ferredoxin-NADP reductase (spots 46 and 47), a putative enoyl-CoA hydratase (spot 51) and an alternative oxidase 2 mitochondrial precursor (spot 55).

2.2.3. Protein changes in the resistant Ps 624 genotype in response to O. crenata inoculation

For the resistant genotype, a lesser number of protein changes were observed in response to *O. crenata* inoculation with only 19 modifications recorded (Fig. 4 and Table 6). Among them, 15 concerned an increase and 3 a decrease in protein amount. As in the case of the susceptible Messire genotype, one protein looked to be newly induced. Among the MALDI-TOF analysed spots, homologies were found for eight proteins (Table 7). Identified proteins included two glutamine root synthetases (spots 61 and 62), an isovaleryl-CoA dehydrogenase (spot 63), three peroxidase 43 precursors (spots 64, 68 and 75) and two glucan endo-1,3-β-glucosidase precursors (spots 67 and 69).

3. Discussion

Screening for *O. crenata* infection performed under field conditions with different pea cultivars and *P. sativum* accessions has revealed the existence of variability in the number of emerged broomrape shoots among genotypes, providing the presence of some level of

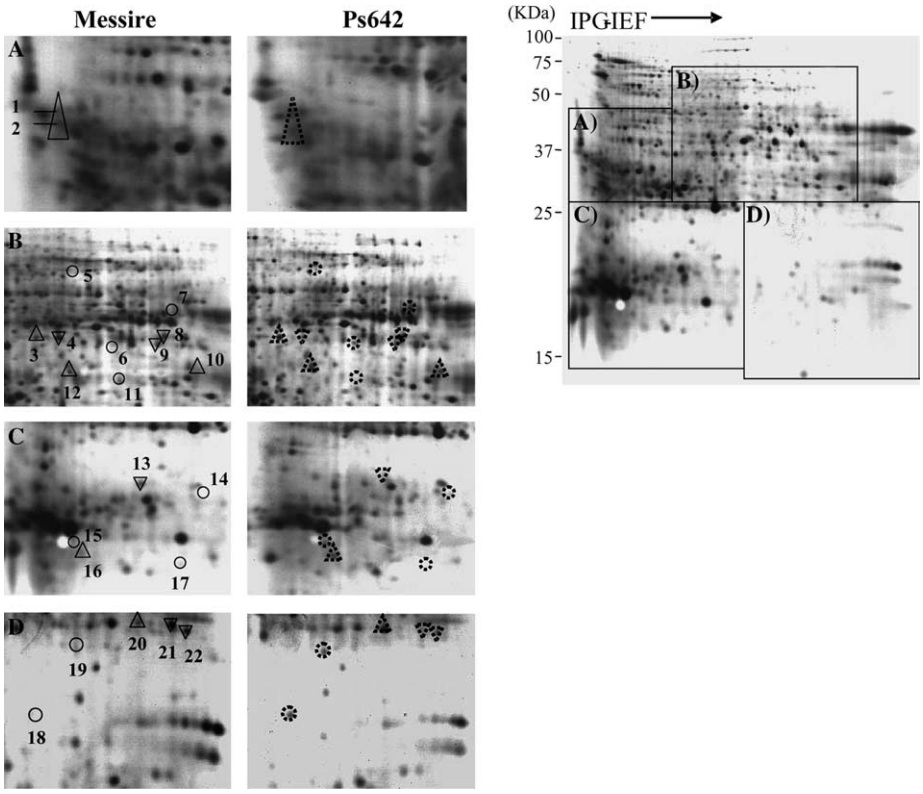

Fig. 2. Comparison of the silver-stained two-dimensional gel electrophoresis root protein between non-infected Messire and Ps 624. One hundred μg proteins were separated by IEF (pH 3–10 linear gradient) and SDS–PAGE (12% vertical slab gels). Numbers indicate protein M_w markers. The gel (right), corresponding to the non-infected Messire root extract, was divided into areas A, B, C and D, being displayed in detail (left). Circles indicate new spots, triangles and reversed triangles the spots with increased and decreased intensity, respectively. Spot numbers and their respective pI and M_w are indicated in Table 2.

resistance, although immunity has so far not been identified (Rubiales et al., 2003b, 2004). Plant resistance to broomrape operates at the early stages of the parasite biological cycle with different genotypes presenting alternative mechanisms, including low induction of germination, host tissue darkening at the infection site or reduced establishment of tubercles (Rubiales et al., 2004). In the present work, we reported difference in their resistance to *O. crenata* between two pea genotypes, with Messire being more susceptible than Ps 624. To investigate the *O. crenata* pea rot interaction in genotypes differing in *Orobanche* resistance, a proteomic approach was performed. Proteins differentially expressed between susceptible and resistant pea roots, either *O. crenata*-infected or not, were investigated by using two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry.

Out of the 79 analysed proteins, only 21 could be matched against SWISS-PROT, NCBI and MSDB databases (Tables 3, 5, 7). Most of the identified proteins corresponded to pea specific matches (18), with high matching score and number of matched peptides, and the remaining to *A. thaliana* (peroxidase), *Solanum tuberosum* (fructokinase), *Oryza sativa* (putative enoyl-CoA hydratase), and soybean (mitochondrial alternative oxidase 2), with the lowest matching score and matched peptides. In all the pea-specific matches, theoretical and experimental p*I* and M_w were in good agreement, encouraging confidence in the identifications. That was not the case for the cross species identifications (spots 33, 51, 64, 68 and 75), which should therefore be interpreted with caution. It has been shown that PMF should only be undertaken against sequence data derived from the organism of interest (Mathesius et al., 2001). Recently, cross species matches were reported as being possible in the case of closely relatives and conserved proteins (Heazlewood and Millar, 2003). However, the method efficiency may also depend on the organisms analysed. In our experimental biological system, an increase in successful identifications using MALDI-TOF analysis requires the availability of more genomic data on both *P. sativum* and *O. crenata*.

Our study revealed both qualitative as well as quantitative changes among genotypes and treatments. Quantitative changes, expressed as relative normalized volumes, ranged from 0.2 to 4.7 among genotypes (Messire/Ps 642), from 0.2 to 1.9 among Messire treatments (inoculated/control), and from 0.3 to 3.3 among Ps 642 treatments (inoculated/control). Assuming values of around 20% for the analytical variance associated to

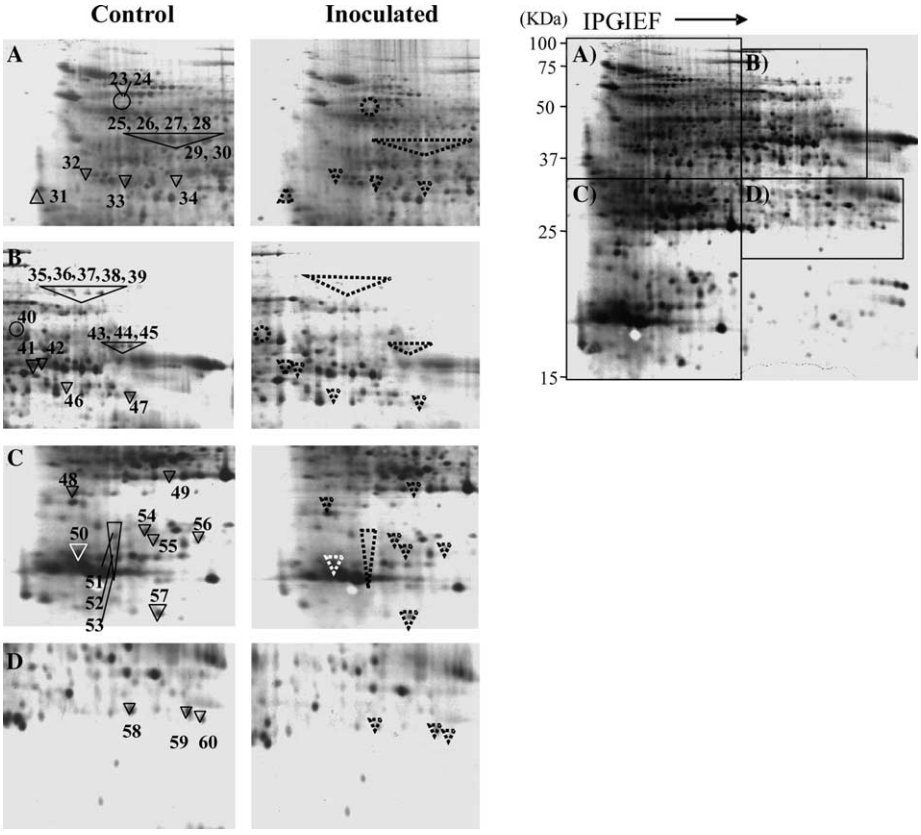

Fig. 3. Comparison of the silver-stained two-dimensional gel electrophoresis root protein between non-infected and *O. crenata*-infected Messire pea genotype. One hundred μg proteins were separated by IEF (pH 3–10 linear gradient) and SDS–PAGE (12% vertical slab gels). Numbers indicate protein M_w markers. The gel (right), corresponding to the non-infected Messire root extract, was divided into areas A, B, C, and D, being displayed in detail (left). Circles indicate new spots, triangles and reversed triangles the spots with increased and decreased intensity, respectively. Spot numbers and their respective p*I* and M_w are indicated in Table 4.

the technique (Asirvatham et al., 2002; Jorge et al., 2004) only ratios higher than 1.2 or lower than 0.8 could be considered as biologically significant. In our case, 61 proteins out of the 79 listed (Tables 2, 4 and 6) fall within this range.

Between the two non-infected pea genotypes which, interestingly, protein difference matched to several proteins known to be produced by plants at higher levels or more rapidly following infections or stresses. β-1,3-glucanase and chitinase are typical pathogenesis-related proteins involved in the resistance against various microorganisms (Mauch et al., 1998). Irrespective of *O. crenata* infection, it could be suggested that a higher constitutive level in both enzymes in the resistant genotype could help the plant in priming defence reactions against pathogens more rapidly in this genotype. ABA-responsive genes encode proteins synthesized during late seed development (Iturriaga et al., 1994) and are typical stress proteins induced under drought or salinity conditions (Ozturk et al., 2002). As a consequence of water input through the host plant, *Orobanche* and other parasitic plants cause water deficits, so the induction of water stress genes could be very important as a defensive strategy. Plant cysteine proteases play a role in a number of processes, such as a nutritional in reserve development and fruit ripening, degradation of storage proteins in germinating seeds, activation of proenzymes, and degradation of defective proteins. Besides this, they have been implicated in the defence against predators through the degradation of exogenous proteins (Boller, 1986). del Pozo and Lam (1998) reported that caspase-like plant proteases participate in the cell death during the hypersensitive response. Finally, the profucosidase is the only protein spot identified that is present in a larger amount in Messire than in Ps 624. α-Fucosidases degrade fucosyl residues from xyloglucans, which are proposed to regulate the plant cell growth rate (Fry, 1989). A plant cell wall α-fucosidase has been purified from pea epicotyls (Augur et al., 1993) and, in a recent report (Repetto et al., 2003), a profucosidase has been identified by 2-DE and LC-MS/MS from pea root extracts.

The protein patterns of the two genotypes were then further analysed in response to the parasitic infection. Following *O. crenata* root infection of the susceptible Messire genotype, we observed a decrease in protein

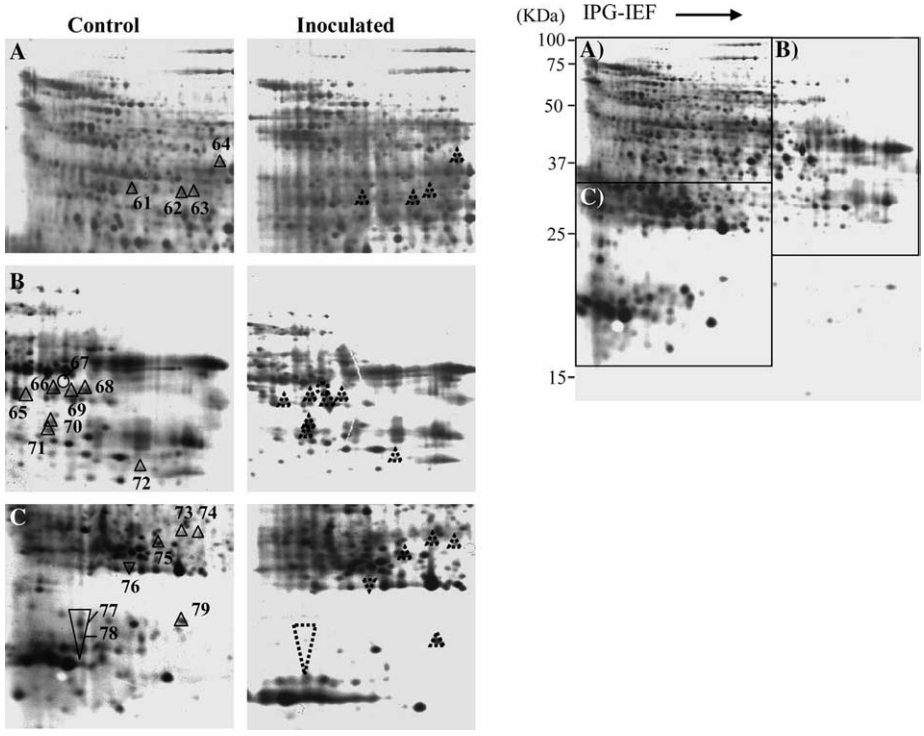

Fig. 4. Comparison of the silver-stained two-dimensional gel electrophoresis root protein between non-infected and *O. crenata*-infected Ps 624 pea genotype. One hundred μg proteins were separated by IEF (pH 3–10 linear gradient) and SDS–PAGE (12% vertical slab gels). Numbers indicate protein M_w markers. The gel (right), corresponding to the non-infected Ps 624 root extract, was divided into areas A, B, and C, being displayed in detail (left). Circles indicate new spots, triangles and reversed triangles the spots with increased and decreased intensity, respectively. Spot numbers and their respective p*I* and M_w are indicated in Table 6.

abundance for 22 spots, among which, enzymes belonging to carbohydrate metabolism, nitrogen assimilation and mitochondrial transport pathway could be identified. Plant fructokinases are the gateway to fructose metabolism, playing an important regulatory role of the flux of carbon through carbohydrate metabolism. In addition, the possible function of fructokinases as sugar sensors has been speculated (Pego and Smeekens, 2000). In plants, it exists as organelle (cytosolic or plastidic) or tissue-specific isoforms, feeding fructose-6-phosphate to the glycolysis, pentose-phosphate or starch synthesis pathways. In tomato, its expression is controlled at the transcriptional level with mRNA primarily expressed in young fruit and mature root tissue (MartinezBarajas et al., 1997). Fructose-1,6-bisphosphate aldolase catalyses one of the reactions of the glycolytic/gluconeogenic pathways, the cleavage of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Transformed potato plants showing low levels of aldolase activity displayed a growth inhibition (Haake et al., 1999). Alternative oxidase is a nuclear-encoded mitochondrial protein mediating the non-proton transfer of electrons from the ubiquinone pool to molecular oxygen. In soybean, it is encoded by a multigenic family, with genes differentially expressed in a tissue-dependent manner (Finnegan et al., 1997). Its function in plants during normal vegetative growth and development is still a matter of speculation. It has been shown to be induced by several stresses and it was proposed that non-coupled oxidation could regulate the level of reduction of the components of the electron transport chain and the rate of one-electron reduction of oxygen, thereby affecting the rate of formation of reactive oxygen species (Popov, 2003). The ferredoxin-$NADP^+$ oxidoreductase is a plastidial enzyme that catalyses the reduction of ferredoxin by NADPH in heterotrophic plant cells, this reaction providing the reducer amount necessary for the assimilation of inorganic nitrogen. Inorganic nitrogen assimilation by plants used to be limiting for plant growth.

Although a more complete success into the protein identifications would have helped us to better interpret the behavior of the susceptible *O. crenata*-infected genotype, from the present data, it is tempting to speculate that an inhibition of the root carbohydrate oxidation pathways occurred when roots were challenged with the parasite. This subsequent decrease in the energy production may also affect the nitrogen assimilation pathway. Under stress conditions, root growth is generally inhibited, a feature commonly attributed to a reduction of the photosynthetate availability and correlated with

Table 2
Protein spots that showed qualitative or quantitative changes in roots of the control, non-infected, Messire and Ps 624 accessions

Spot number	Gel area[a]	M_w (kDa)[b]	pI[b]	Relative normalized volume[c] Messire/Ps 642
1	A	33.8	3.94	0.9
2	A	31.8	3.95	0.6
3	B	39.2	5.89	0.6
4	B	39.2	6.17	4.7
5	B	52.2	6.34	0.0
6	B	38.0	6.92	0.0
7	B	44.0	7.73	0.0
8	B	39.2	7.63	2.2
9	B	37.9	7.53	1.9
10	B	34.2	8.18	0.2
11	B	32.7	6.99	∞[d]
12	B	34.8	6.33	0.7
13	C	22.7	5.51	2.0
14	C	21.8	6.41	0.0
15	C	18.2	4.60	0.0
16	C	17.5	4.75	0.3
17	C	17.3	6.06	∞[d]
18	D	22.4	7.24	0.0
19	D	25.9	7.62	0.0
20	D	28.2	8.39	0.5
21	D	27.5	9.00	2.4
22	D	28.0	8.81	4.5

Only those changes consistently manifested in all the four independent replicates were included.
[a] Localization of spots according to the gel areas defined in Fig. 2.
[b] Molecular masses (M_w) and isoelectric points (pI), as well as normalized volumes were calculated with the PD-Quest Software.
[c] Values are mean of the four independent replicates.
[d] Non-detected in Messire.

an increase in sucrose, glucose and fructose content and a decrease in the hexose-phosphate pool. The effect of *Orobanche* and *Striga* parasitism on host plant growth, photosynthesis, photoassimilate partitioning, and nitrogen metabolism has been extensively studied (Barker et al., 1996; Hibberd et al., 1999). The observed metabolic changes can also reflect either a decrease in the photosynthetic activity occurring in parasite-infected plants and/or a decrease in the availability of the translocated sucrose to the host cells in relation to the sink potential of the installed broomrape.

While the susceptible Messire displayed a decrease in the amount of 22 protein spots in response to *O. crenata*, an increase in 15 spot abundance was observed for the resistant genotype Ps 624 upon infection. Among them, the eight proteins successfully identified following the PMF search corresponded, on the one hand, to nitrogen metabolism enzymes (glutamine synthetase, isovaleryl-CoA dehydrogenase) and, on the other hand, to typical proteins of defence against pathogens (β-1,3-glucanase, peroxidase precursor). Glutamine synthetase catalyses the ATP-dependent assimilation of ammonium into glutamine, using glutamate as substrate. In plants, it exists as two main isoforms, a cytosolic and a chloroplast or plastidic one (Woodall and Forde, 1996). The increase in the glutamine synthetase in inoculated plants could be related to the reported effect of nitrogen on the level of infection by parasitic plants (Pres, 1994). Isovaleryl-CoA dehydrogenase catalyses the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA in the leucine catabolism pathway. It has been cloned from pea, although physiological data have not been provided (Reinard et al., 2000).

Concerning the plant defence response category of proteins, the β-1,3-glucanase whose level is increased in the resistant Ps 624 in response to *O. crenata* inoculation, was detected above only in the non-inoculated Ps 624 roots. Only a few reports have dealt with the occurrence of pathogenesis-related proteins in the plant/*Orobanche* interactions (Joel and Portnoy, 1998). In our case, it can be suggested that the β-1,3-glucanase is involved in the defence against *O. crenata* infection. Regarding peroxidases, they belong to a large family of enzymes able to oxidize various substrates in the presence of H_2O_2. In higher plants, the number of peroxidase genes and their corresponding proteins is extremely high. They are ascribed with a variety of functional roles, including lignification and cell wall phenol deposition, suberisation, hormone catabolism, developmental related processes, defence against pathogens and

Table 3
Peptide mass fingerprinting analysis and identification of differential expressed protein spots between non-infected, pea roots from Messire and Ps 624 accessions

Spot No.	Homologous protein	Species (Swiss-Prot accession No)	M_w/pI[a] Experimental (theoretical)	Matched peptides[b]	Sequence coverage (%)
1	Cysteine proteinase (E.C.3.4.22.)	*Pisum sativum* Q41064	33.8/3.9 (51.2/6.1)	11	25
6	Glucan endo-1,3-β glucosidase precursor (E.C.3.2.1.39)	*Pisum sativum* Q03467	38.0/6.9 (41.0/6.2)	15	41
12	Endochitinase A2 precursor (E.C.3.2.1.14)	*Pisum sativum* P21226	34.8/6.3 (34.7/7.3)	10	32
13	Profucosidase (E.C.3.2.1.51)	*Pisum sativum* O82711	22.7/5.5 (23.6/6.2)	7	24
15	ABA-responsive protein	*Pisum sativum* Q06931	18.2/4.6 (16.6/5.1)	5	49

[a] Experimental molecular mass (M_w, kDa) and pI of proteins calculated with PD-Quest Software (BioRad) from standards proteins coelectrophoresed with root extracts; theoretical M_r and pI of the homologous protein in database.
[b] Number of matched peptides.

Table 4
Protein spots that showed qualitative or quantitative changes in roots of the Messire accession in response to the *O. crenata* inoculation

Spot number	Gel area[a]	M_w (kDa)[b]	p*I*[b]	Relative normalized volume[c] inoculated/control
23	A	59.2	4.93	∞[d]
24	A	59.0	4.79	∞[d]
25	A	47.8	5.14	1.7
26	A	46.7	5.26	0.5
27	A	46.6	5.47	0.5
28	A	47.8	5.66	0.2
29	A	46.6	5.92	0.9
30	A	46.7	6.20	1.1
31	A	34.9	3.75	1.9
32	A	38.9	4.59	0.9
33	A	37.4	5.03	0.8
34	A	37.3	5.77	0.9
35	B	66.3	7.30	0.7
36	B	63.6	7.34	0.7
37	B	65.7	7.57	0.9
38	B	64.2	7.83	0.7
39	B	64.3	8.02	0.9
40	B	49.2	6.67	∞[d]
41	B	40.2	7.00	0.9
42	B	40.5	7.13	0.8
43	B	44.9	8.20	0.7
44	B	45.2	8.34	0.8
45	B	45.1	8.45	0.9
46	B	36.0	7.51	0.6
47	B	35.0	8.46	0.4
48	C	24.1	4.35	0.9
49	C	25.5	5.66	0.6
50	C	19.9	4.42	0.5
51	C	21.0	4.91	0.3
52	C	20.3	4.94	0.4
53	C	19.8	5.00	0.8
54	C	21.4	5.34	0.7
55	C	20.7	5.48	0.9
56	C	21.0	6.10	0.9
57	C	16.4	5.52	0.9
58	D	26.3	8.27	0.8
59	D	26.1	9.03	0.9
60	D	25.7	9.21	0.9

Only changes consistently detected in all the four independent replicates were included.
[a] Localization of spots according to the gel areas defined in Fig. 3.
[b] Molecular masses (M_w) and isoelectric points (p*I*), as well as normalized volumes were calculated with the PD-Quest Software.
[c] Mean value of the four independent replicates.
[d] Non-detected in control Messire.

response to other stresses (Penel et al., 1992). An increase in lignification and peroxidase activity has been observed in *O. aegyptiaca* infected vetch plants (Goldwasser et al., 1999). In addition, a peroxidase mRNA accumulated in *Arabidopsis* plants during the first 24 h after infection with *O. ramose* (Vieira dos Santos et al., 2003). In previous studies with different pea genotypes, we have observed that most of the *Orobanche*-resistant genotypes showed a higher constitutive peroxidase activity than susceptible ones (Pérez de Luque, 2002). In our work, because an increase in peroxidase proteins was only observed in the infected resistant genotype, it can be suggested that peroxidase proteins play a role in pea plants resistance against *O. crenata*.

To conclude, by using a proteomic approach, we reported here for the first time the identification of proteins differentially expressed between two *O. crenata*-infected pea genotypes differing in their *Orobanche* sensitivity. In response to the parasite infection, two clear and opposite tendencies were observed between the Messire and Ps 624 genotypes. Whereas *O. crenata* infection led to a decrease in the amount of proteins matching with enzymes involved in the energetic metabolism in the susceptible genotype, it gave rise to an increase in the abundance of proteins identified as defence proteins and glutamine synthetases in the resistant one. The fact that two metabolic pathways (carbon and nitrogen) seemed to be affected in relation to susceptibility/resistance of the pea genotypes opens up new possibilities to better understand the re-direction of host assimilates from host sinks to the parasite. In the present work, 28% of the analysed proteins were identified following MALDI-TOF mass spectrometry. Increase in the identification rate could be achieved following tandem mass spectrometry together with the use of new bioinformatic tools (Liska and Shevchenko, 2003). This will allow us to obtain a more global view of the pea roots/*O. crenata* interactions.

4. Experimental

4.1. Plant material, growth conditions and inoculation

The *P. sativum* genotypes Messire and Ps 624 were utilized. *Orobanche crenata* seeds were collected from field infected pea plants and stored in darkness at room temperature. Germination tests with the synthetic germination stimulant GR-24 were performed as previously described (Pérez-de-Luque et al., 2000). The germination percentage of the broomrape population used reached about 60%.

Pot experiments were carried out in a greenhouse under natural light conditions and temperature 20 ± 5 °C. Ten plants per accession were grown individually in pots filled with 1 l of vermiculite mixed with 30 mg (about 9000 seeds) of broomrape seeds. Plants were watered weekly with 50 ml/pot of Hoagland nutritive solution. Three months after sowing the plants were harvested with the roots gently washed with water, the number of emerged shoots and tubercles counted and their developmental stage recorded (Ter Borg et al., 1994): (1) tubercles smaller than 2 mm; (2) tubercles bigger than 2 mm but without root formation; (3) tubercles with crown-root; (4) sprout already visible re-

Table 5
Peptide mass fingerprinting analyses and identification of differential expressed protein spots in infected Messire accessions

Spot No.	Homologous protein	Species (Swiss-Prot accession No)	M_w/pI[a] Experimental (theoretical)	Matched peptides[b]	Sequence coverage (%)
33	Fructokinase (E.C.2.7.1.4)	*Solanum tuberosum* P37829	37.4/5.0 (33.8/5.5)	7	17
41	Fructose-bisphosphate aldolase, cytoplasmic isozyme 2 (E.C.4.1.2.13)	*Pisum sativum* P46257	40.2/7.0 (38.5/6.8)	11	41
42	Fructose-bisphosphate aldolase, cytoplasmic isozyme 2 (E.C.4.1.2.13)	*Pisum sativum* P46257	40.5/7.1 (38.5/6.8)	9	28
46	Ferredoxin-NADP reductase, root isozyme (E.C.1.18.1.2)	*Pisum sativum* Q41014	36.0/7.5 (42.3/8.8)	12	33
47	Ferredoxin-NADP reductase, root isozyme (E.C.1.18.1.2)	*Pisum sativum* Q41014	35.1/8.5 (42.3/8.8)	28	65
51	Putative enoyl-CoA-hydratase	*Oryza sativa* Q9AYM2	21.0/4.9 (40.7/10.8)	24	64
55	Alternative oxidase 2 mitochondrial precursor	Soybean Q41266	21.4/5.3 (38.1/9.4)	4	16

[a] Experimental molecular mass (M_w, in kDa) and pI of proteins calculated with PD-Quest Software (BioRad) from standards proteins coelectrophoresed with root extracts; theoretical M_r and pI of the homologous protein in database.
[b] Number of matched peptides.

Table 6
Protein spots that show qualitative or quantitative changes in roots of the Ps 624 accession in response to *O. crenata* inoculation

Spot number	Gel area[a]	M_w (kDa)[b]	pI[b]	Normalized volume[c] inoculated/control
61	A	43.4	5.57	1.2
62	A	44.0	6.39	1.8
63	A	44.8	6.53	1.2
64	A	48.5	6.77	1.1
65	B	37.8	7.47	3.3
66	B	38.2	7.76	1.4
67	B	39.3	7.89	∞[d]
68	B	38.1	8.09	2.3
69	B	36.8	7.95	1.8
70	B	34.2	7.72	2.9
71	B	33.3	7.69	2.3
72	B	29.4	8.68	1.6
73	C	32.8	6.41	1.5
74	C	32.6	6.67	1.1
75	C	29.4	5.60	1.3
76	C	32.6	6.20	0.7
77	C	23.2	4.84	0.3
78	C	22.0	4.86	0.7
79	C	23.3	6.41	1.1

Only changes consistently detected in all the four independent replicates were included.
[a] Localization of spots according to the gel areas defined in Fig. 4.
[b] Molecular masses (M_w) and isoelectric point (pI), as well as normalized volumes were calculated with the PD-Quest Software.
[c] Mean values of the four independent replicates.
[d] Non-detected in control Ps 624.

maining underground; (5) shoot emergence; (6) flowering; (7) setting of seeds.

The Petri dish infection bioassay was performed in a plant growth chamber under controlled conditions. Plants were grown individually and infected as described by Rubiales et al. (2003a). Pea seeds were germinated in Petri dishes on wet fiber papers and kept in the dark at 20 °C for five days. When root reached 4–5 cm length, plants were transferred to new dishes (∅15 cm) with perlite and glass fiber papers (Whatmann GF/A). *O. crenata* seeds (8 mg) were previously spread on the paper, after being disinfected with ethanol (70%, 30 s), bleach (20%, 20 min) and placed in darkness at 20 °C for 10 days. Dishes were sealed with parafilm, covered with aluminium foil to prevent roots and broomrape seeds from the light, placed vertically, the germinating host plant upwards, in trays with Hoagland nutrient solution and kept in the growing chamber at 20 °C with 14 h light. Forty-five days later the number of tubercles per plant were counted and classified according to the above mentioned 1–7 scale. For proteomic analysis, the dish experiment was repeated with the modification that 5 ml (10 mg l^{-1}) of the strigol analogue GR-24 (kindly provided by Dr. Zwanenburg) was added all over the surface to ensure broomrape seed germination. Pea roots from control (non-infected) and infected plants were sampled 45 days after transplanting. *Orobanche* seeds and tubercles were removed, root tissue abundantly washed with tap water, distilled water, blot dried with filter paper, frozen in liquid nitrogen, stored at −70 °C and lyophilized prior to the analysis.

4.2. Protein extraction and two-dimensional gel electrophoresis

Proteins from lyophilized root tissues (1.2–4 g root fresh weight) were phenol-extracted according to Dumas-Gaudot et al. (2004). Following overnight ammonium acetate precipitation, the pellet recovered by centrifugation was rinsed with cold methanol and acetone, dried under nitrogen gas and resuspended in 400 μl of solubilization buffer containing 9 M urea, 4% (w/v) CHAPS, 0.5% (v/v) TritonX100, 100 mM DTT and 2%

Table 7
Peptide mass fingerprinting analyses of differential expressed protein spots in *O. crenata* infected Ps 624 accessions

Spot number	Homologous protein	Species (Swiss-Prot accession No)	M_w/pI[a] Experimental (theoretical)	Matched peptides[b]	Sequence coverage (%)
61	Glutamine synthetase root isozyme B (E.C.6.3.1.2)	*Pisum sativum* Q43066	43.4/5.6 (39.3/6.0)	13	41
62	Glutamine synthetase root isozyme A (E.C.6.3.1.2)	*Pisum sativum* P07694	44.0/6.4 (39.3/6.1)	9	35
	Glutamine synthetase root isozyme B (E.C.6.3.1.2)	*Pisum sativum* Q43066	44.0/6.4 (39.3/6.0)	9	35
63	Isovaleryl-CoA-dehydrogenase (E.C.1.3.99.10)	*Pisum sativum* Q9SM61	44.8/6.5 (44.5/6.3)	12	36
64	Peroxidase 43 precursor (E.C.1.11.1.7)	*Arabidopsis thaliana* Q9SZH2	48.5/6.8 (35.4/6.7)	5	16
67	Glucan endo-1,3-β glucosidase precursor (E.C.3.2.1.39)	*Pisum sativum* Q03467	39.3/7.9 (41.0/6.2)	11	31
68	Peroxidase 43 precursor (E.C.1.11.1.7)	*Arabidopsis thaliana* Q9SZH2	38.1/8.1 (35.4/6.7)	***5***	15
69	Glucan endo-1,3-β glucosidase precursor (E.C.3.2.1.39)	*Pisum sativum* Q03467	36.8/7.9 (41.0/6.2)	16	33
75	Peroxidase 43 precursor (E.C.1.11.1.7)	*Arabidopsis thaliana* Q9SZH2	31.5/6.0 (35.4/6.7)	7	15
79	Profucosidase (E.C.3.2.1.51)	*Pisum sativum* O82711	23.3/6.4 (23.6/6.2)	8	24

[a] Experimental molecular mass (M_w, in kDa) and pI of proteins calculated with PD-Quest Software (BioRad) from standards proteins coelectrophoresed with root extracts; theoretical M_r and pI of the homologous protein in database.
[b] Number of matched peptides.

(v/v) IPG buffer pH 3–10 (Amersham Biosciences), shaking the mixture for 2 h. Lipids and nucleic acids were removed by ultracentrifuging during 30 min (Beckman Airfuge, 30 Psi) and protein content in the supernatant was quantified by the method of Bradford, as modified by Ramagli and Rodriguez (1985), using ovalbumin as a standard. Samples were stored at −20 °C before electrophoresis.

Four replicates of 2-DE gels were performed for each independent extracted sample. Precast 17 cm linear pH 3–10 (BioRad) were rehydrated overnight with 350 μl of buffer containing 8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 2% (v/v) IPG Buffer pH 3–10 and bromophenol blue. For analytical separations, 100 μg of protein were loaded at the cathodic end of strips and electrofocussed at 20 °C for 56 kV h. For micropreparative separations, 500 μg of proteins were focussed for 84 kV h. A gradually increasing voltage was used in both cases. Strips were loaded onto a Multiphor II horizontal electrophoresis system (Amersham Pharmacia Biotech). After isoelectric focusing, IPG strips were equilibrated by soaking first for 10 min in 50 mM Tris–HCl buffer, pH 8.8, 6 M urea, 2% SDS, 34% glycerol, solution, containing 1% DTT, and then for 10 min in the solution containing 5% iodoacetamide. Strips were then transferred onto vertical slab 12% SDS–polyacrylamide gels and electrophoresis was run at 10 °C for 1 h at 35 V, and then at 100 V for about 14 h, until the dye front reached the bottom of the gel.

Analytical gels were silver-stained while the micropreparative ones were stained with Coomasie Brilliant Blue G-250 (BioRad) according to the procedure of Mathesius et al. (2001). Gel images were obtained by a GS-800 imaging densitometer (BioRad), and analysed with the PD-Quest™ software (BioRad), by using a 10-fold over background as minimum criteria for presence/absence. The analysis was re-evaluated by visual inspection, focusing on those spots most drastically altered in the treatments and plant genotypes.

4.3. MALDI-TOF mass spectrometry and database searching

Spots from Coomassie stained gels were manually excised and washed with 100 μl of 50% acetonitril/50 mM hydrogenocarbonate pH 8 solution until destaining. Gel pieces were then dehydrated with acetonitrile and dried under vacuum centrifugation for 30 min. Rehydration in 10 μl of 50 mM ammonium hydrogenocarbonate pH 8 containing 0.5 μg of Promega porcine trypsin was performed, with digestion carried out overnight (16–18 h) at 37 °C. Peptide fragments from digested proteins were then subjected to MALDI-TOF-MS (Applied Biosystems, Voyager DE super STR) for peptide fingerprinting. This instrument is equipped with N_2 laser (337 nm, Laser of 20 Hz). Samples were acquired in reflectron mode (positive mode) with a delay of extraction time of 130 ns. Internal calibration was performed using trypsin peptide masses in a mass range of 500–5000 Da.

Peptide masses were used to search SWISS-PROT (Protein Prospector, http://www.expasy.ch/tools/pepti-

dent.html), NCBI and MSDB (MASCOT, http://www.matrixscience.com) databases. Ranges were 1.0 for p*I* and 20% for molecular weights. Mass accuracy was 20 ppm. Possible modifications were carboxyamidomethyl cysteines. Up to 1 missed trypsin cleavage was allowed.

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